

Evaluation of *Ginkgo biloba* as Alternative Medicine on Ova-Induced Eotaxin and Eosinophilia in Asthmatic Lung

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Abstract: *Ginkgo biloba* is an ancient plant; leaves of this plant have been used in asthma and bronchitis for many centuries. A model of lung eosinophilia based on the repeated exposure of mice to allergen ovalbumin (OVA). This model was used to investigate the effect of *Ginkgo biloba* leaf powder extract (GBE) on airway inflammation and asthmatic lung. Asthma is a chronic disease characterized by reversible airway obstruction. Airway inflammation is the key factor in the pathogenesis of asthma and current strategies for the management focus on suppressing airway inflammation. Eotaxin is an eosinophils specific chemo attractant that has been recently identified in rodent models of asthma. Cytokines IL-4 or IL-13, especially in combination with tumor necrosis factor alpha (TNF- α), resulted in substantial release of the potent eosinophil chemoattractant factor, eotaxin. Eosinophilic leukocytes accumulate in high number in the lungs of asthmatic mouse and are, believed to be important in the pathogenesis of asthma. To observe the effects of GBE on asthmatic mice, leukocytes and eosinophils migration were counted in bronchoalveolar lavage fluid (BALF). The production of IL-4, IL-13, and TNF- α were estimated in BALF. Moreover, the mRNA expression of eotaxin was analyzed in BALF by RT-PCR. The present study showed that, there was correlation between eotaxin level and eosinophils infiltration in the allergen OVA exposure group. The results revealed that, GBE markedly inhibit cells migration in BALF, in addition to, reduce the levels of IL-4, IL-13, and TNF- α . This reduction extends to the mRNA expression of eotaxin in BALF. The present results demonstrated that GBE can decrease the severity of asthma not only by suppressing eotaxin but also by inhibiting cytokines production. In this research, we address the question of whether *Ginkgo biloba* leaf powder extract (GBE) can inhibit the eosinophils infiltration, eotaxin and cytokine production in asthmatic lung.

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1. Introduction

Many patients harbour misgivings about conventional medical treatments for asthma, particularly inhaled corticosteroid treatment (Chan and DeBruyne, 2002). There is a need for development of additional effective treatments with fewer Side effects. Recently, there has been a surge in interest in herbal medicine, possibly because they have fewer side effects than current therapy (Bielory and Lupoli 1999; Hocaoglu *et al.*, 2012).

Ginkgo biloba extract (GBE) is an ancient plant, its standardized extract of leaves has been extensively used in diseases of cardio vascular system and cerebro-vascular system, *Ginkgo biloba* extract has been mentioned in the traditional Chinese pharmacopoeia, and Chinese has used ginkgo leaves for asthma and bronchitis for many centuries. (Hu *et al.*, 2000; Tang, 2012). *Ginkgo biloba* extract (GBE), has been used therapeutically. It is a known inhibitor of platelet activating factor (PAF), which is important in the pathogenesis of asthma. (Chu *et al.*, 2011). This herbal medicine comes in the form of an herbal tea and capsule supplement form.

Asthma is a heterogeneous disease in which various cytokines orchestrate airway inflammation (Babu *et al.*, 2004). Eosinophils play an important role in allergic disorders such as Allergic asthma (Arm *et al.*, 1997). Upon allergen challenge, eosinophils migrate from the peripheral blood into allergic inflammatory tissues and are subsequently activated in bronchoalveolar lavage fluid obtained from asthmatic patients, toxic granule products, such as eosinophils cationic protein, can be detected (Woolley *et al.*, 1995). The release of these toxic eosinophil cationic proteins and major basic protein (degranulation) can result in damage of the respiratory epithelium, leading to airway hyper-responsiveness (Bracke *et al.*, 2000).

Eotaxin, a chemokine, likely plays an important role in the eosinophilia of asthma. Eotaxin was first identified as important chemo-attractant for eosinophils in antigen-sensitized and challenged guinea pig lungs (Jose *et al.*, 1994). Subsequent experiments in murine models of airway inflammation confirmed these findings (Gonzalo *et*

al. , 1996). Furthermore, eotaxin is up regulated in bronchoalveolar lavage fluid and airways of asthmatic patients (Lamkhioed *et al.* , 1997). Expression of eotaxin mRNA was recently shown to be up-regulated after segmental allergen challenge in subjects with atopic asthma (Lilly *et al.* , 2001). Despite the importance of eotaxin recruitment, the regulation of eotaxin expression in the asthmatic airway remains to be established. *In vitro* studies on epithelial and endothelial cells have demonstrated that the proinflammatory cytokine TNF- α increases eotaxin expression (Gracia-Zepeda *et al.* , 1996). Many cell types in the lung appear to be capable of synthesizing eotaxin (e.g airway vascular endothelial cells and macrophages as well as eosinophils themselves) (Moore *et al.*, 2001) . Eosinophilic leukocytes accumulate in high numbers in the lungs of asthmatic patients, and are believed to be important in the pathogenesis of asthma. A potent eosinophil chemo-attractant, eotaxin is produced in the asthmatic lung (Conroy and Williams, 2001) . This small protein, is synthesized by a number of different cell types, and is stimulated by IL-4 and IL-13, which are produced by T-helper (Th)2 lymphocytes. Low molecular weight compounds have been developed that can block the eotaxin receptor, and prevent stimulation by eotaxin. This provides the potential for orally available drugs that can prevent eosinophil recruitment into the lung and the associated damage and dysfunction (Conroy and Williams, 2001).

Cytokines IL-13 and IL-4 , which have been implicated in asthma, have been also shown to induce eotaxin expression in dermal and pulmonary fibroblasts and airway epithelial cells (Teran *et al.* , 1999). Animal models also support a role for IL-13 and IL-4 in eotaxin release and eosinophil recruitment. IL-13 has effect on immune cells that are similar to those of the closely related cytokine IL-4. However, IL-13 is suspected to be a more central mediator of the pathological changes induced by allergic inflammation in many tissues (Li *et al.*, 1999 ; Zhu *et al.*, 1999).

Tumor necrosis factor alpha (TNF- α) is a proinflammatory cytokine that has been implicated in the modulation of inflammation in various diseases, including asthma. TNF- α blocking strategies have been an effective therapeutic modality in diseases such as rheumatoid arthritis. Studies were demonstrated that the effect of blocking TNF- α as a possible therapeutic option in patients with severe corticosteroid-dependent asthma (Babu *et al.* ,2004). TNF- α was discovered more than a century ago, and its known roles have extended from within the immune system to include a neuro-inflammatory

domain in the nervous system (Leung and Cahill,2010).

In this research, we address the question of whether *Ginkgo biloba* leaf powder extract (GBE) can inhibit the eosinophils infiltration, eotaxin and cytokine production in asthmatic lung.

2. Material and Methods

Study materials:

The IL-4, IL-13 and TNF- α kits were purchased from Biosource, Ovalbumin was purchased from sigma-Aldrich St. Louis, Mo, USA.

Ginkgo biloba capsule was purchased from Pharaonia Pharmaceutical (Pharo pharma).

Trizol reagent and RT-PCR reagents are from SuperScript Rnase H-RT Kit/Gibco / BR).

Experimental Animals

Pathogen free 6 to 9 weeks, male BALB/c mice, weighing 25-35g, were purchased from Theodor Bilharz Research Institute, Al-Giza Egypt, and maintained in a pathogen-free animal laboratory. They were kept in hygienic cages and at temperature rooms of 20-25°C ,12 hour light/12 hour dark cycle with food and water available *ad-libitum*.

Study Groups:

Twenty mice were divided into four groups:

- **Group I**, OVA- challenged.
- **Group II**, OVA- challenged Plus *Ginkgo biloba*.
- **Group III**, control group treated with GBE, mice received *Ginkgo biloba* 40mg/kg dissolved in saline .The extract was administered by intraperitoneal administration at 1h before the OVA challenge on days 25-27.
- **Group IV**, control group, considered as negative control.

Each group including five mice.

Antigen sensitization, challenge protocol and GBE administration as described by Chu *et al.* (2011)

- The mice were sensitized via two intraperitoneal injections, on day 0 and day 14 of the experiment, with 0.2ml saline containing 20 μ g ovalbumin adsorbed in 0.4mg aluminum hydroxide (Alum.) as adjuvant .On days 25-27, mice were anesthetized with 0.2 ml i.p of ketamine (0.44mg/ml) before receiving an intranasal dose of 100 μ g ovalbumin in a 50 μ l volume of saline.

OVA-challenged group received 40 mg/kg *Ginkgo biloba* leaf powder extract as *Ginkgo biloba* capsule, dissolved in saline. The extract was given by intraperitoneal administration at 1h before OVA challenge on days 25-27. Mice without OVA exposure (control-GBE) group received the same dose of GBE on days 25-27 (Figure 1).

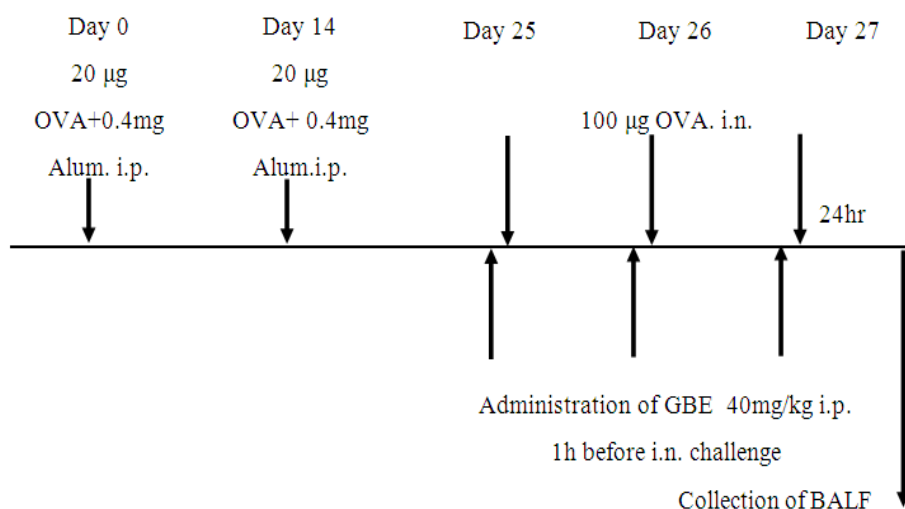


Figure 1. Sensitization, challenge protocol and treatment with *Ginkgo biloba* extract.

Determination of Bronchoalveolar lavage fluid (BALF) cells.

The mouse trachea was cannulated and the lungs were washed 3 times with 1ml of saline. The bronchoalveolar lavage was collected (BAL cells) . 0.05 ml of sample were counted using a hemocytometer and the remaining fluid was immediately centrifuged and the supernatant was collected for cytokine measurements, the pellets were resuspended in 20µl of 10% bovine serum albumin (BSA), then BALF smear were made on glass slides to calculate eosinophil number.

Determination the number of eosinophils,

The slides were stained with Wright-Giemsa, percentage of eosinophils were determined by counting in eight high power fields. Magnification, 40x; total area, 0.5 mm² per area selected and dividing this number by the total number of cells per high power field. This percentage of eosinophil in smear was multiplied by the total number of cells recovered in the lavage fluids according to Gonzalo *et al.* (1996) .

Enzyme linked immune sorbent assay (ELISA) for BALF cytokines analysis.

Cytokine protein levels in BALF; IL-4, IL-13, and TNF- α were measured by ELISA. According to the manufacturer's instructions.

Estimation of mouse eotaxin in BALF by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Twenty-four hour post the last intranasal challenge, bronchoalveolar lavage fluid cells were collected and centrifuged ,the total RNA was extracted

using Trizol reagent, mRNA eotaxin was evaluated according to Szalay *et al.* (1994). cDNA quality was controlled by performing β -actin cDNA.

Statistical analysis

Data were fed to the computer using IBM SPSS software package version 20.0. Quantitative data were described using mean and standard error. Comparison between different groups was analyzed using F-test (ANOVA) and Post Hoc test (Scheffe) for pair wise comparison. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

3.Results

The number of cells migration was significantly higher ($p < 0.001$) in the OVA-challenged group than in the negative control group (Table 1). The OVA challenged GBE group was significantly less in cells number than the OVA-challenged mice group ($p < 0.001$). Contrary, there was no statistically significant difference ($p < 0.001$) between control-GBE group and negative control group (Table 1) .

Furthermore, significant increase ($p < 0.001$) were detected when comparing the levels of Cytokines; IL-4, IL-13, and TNF- α in OVA-challenged group compared to negative control group (Table 1). Concerning, the effect of GBE treatment, the results obtained showed significant decrease in the levels of IL-4, IL-13, and TNF- α compared to OVA-challenged mice group $p < 0.001$ (Table 1).

Table (1):Effect of GBE (40 mg/ kg i.p.) on cell migration; (leukocytes, eosinophils) and cytokines production; IL4, IL3, TNF- α in BALF.

	OVA- challenged (n=5)	OVA- challenged GBE (n=5)	control -GBE (n=5)	control(n=5)	<i>P</i>
Leukocytes $\times 10^6$ /ml	7.04 ^a \pm 0.14	2.58 ^b \pm 0.70	0.30 \pm 0.13	0.34 \pm 0.09	<0.001*
Eosinophils $\times 10^5$ /ml	6.29 ^a \pm 0.60	2.10 ^b \pm 0.30	0.15 \pm 0.07	0.18 \pm 0.07	<0.001*
IL-4 pg/ml	90.78 ^a \pm 2.80	16.54 ^b \pm 3.55	3.02 \pm 1.41	4.14 \pm 1.50	<0.001*
IL-13 pg/ml	99.40 ^a \pm 5.39	17.05 ^b \pm 2.57	5.15 \pm 1.57	6.32 \pm 1.15	<0.001*
TNF- α pg/ml	969.12 ^a \pm 39.74	159.30 ^b \pm 26.99	2.82 \pm 0.99	3.82 \pm 0.83	<0.001*

Data was expressed in mean \pm SE p: p value for F test (ANOVA)
 pair wise between any two groups was assessed using Post Hoc test (Scheffe)
 a: significant with control group b: significant with OVA-challenged group
 *: Statistically significant at $p \leq 0.05$

Effect of GBE on OVA-induced mouse eotaxin

As regards the expression of mRNA eotaxin, the results given in figure 1 showed remarkably increase in OVA-challenged group compared to OVA-challenged GBE group. On the other hand

there was no differences in mRNA expressions eotaxin in both negative control group and control-GBE group (Figure 1).

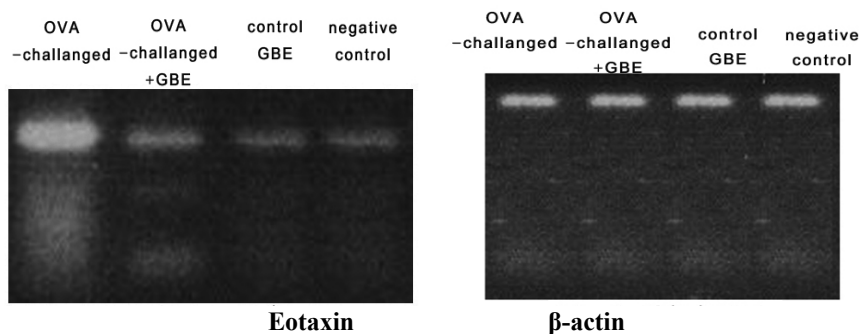


Figure 2. mRNA expression of eotaxin in BALF. The mRNA expression of eotaxin in OVA-challenged GBE group was less than OVA-challenged.

4. Discussion

Asthma is a complex disease characterized by acute and chronic air way inflammation which adversely affects normal lung function, airway hyper-responsiveness, eosinophilia and mucus hypersecretion by goblet cells. (Leonard and Sur, 2002).

Asthma is a heterogeneous disease in which various cytokines orchestrate airway inflammation (Babu *et al.*, 2004)

Many cytokines (IL-4, IL-13) contribute to this inflammation mediated by T-helper cells, which play central roles in the pathogenesis of allergic asthma (Brightling *et al.*, 2002; Bloeman *et al.*, 2007). Airway inflammation is the key factor in the pathogenesis of asthma and current strategies for the management focus on suppressing airway inflammation (Kumar, 2001; Walsh, 2006). The results of the present study come in contact with (Babu *et al.*, 2004; Walsh, 2006; Bloeman *et al.*, 2007) Moreover, the present data demonstrate that GBE significantly reduced leukocytes migration, consequently eosinophils infiltration in BALF of OVA-challenged group.

Eotaxin-induced eosinophil recruitment in asthma. Inhaled allergen activates mast cells and Th2 lymphocytes in the lung to generate the cytokines IL-4, IL-13 and TNF- α . These cytokines stimulate the generation of eotaxin by lung epithelial cells, fibroblast and smooth muscle cells. Eotaxin acting on receptor of eosinophils then stimulates the selective recruitment of these cells from the airway microvessels into the lung tissue (Conroy and Williams, 2001).

Cytokines IL-13 and IL-4, which have been implicated in asthma induced eotaxin expression in dermal and pulmonary fibroblasts and in airway epithelial cells (Teran *et al.*, 1999). Interleukin (IL-13) plays an important role in T-cell differentiation toward a Th2 phenotype and isotype switching of B cells to immunoglobulin IgE production (Horie *et al.*, 1997). IL-13 and tumor necrosis factor- α synergistically induce eotaxin production in human nasal fibroblasts (Terada *et al.*, 2000).

In the present study, pretreatment with GBE resulted in a significant reduction of the production of IL-13 and IL-4 in BALF. This result is in accordance with Jing *et al.* (2008) who demonstrated that extract of

Ginkgo biloba (EGb) blocks mucus hypersecretion of asthmatic rats by inhibiting IL-13.

The eotaxin levels correlated with the number of eosinophils infiltrating the lung tissue, on the other hand, the appearance of significant numbers of eosinophils in the BALF occurred later (12-24 hrs), which may be because the persistence of eotaxin in the airway lumen resulted in the direction of the chemoattractant gradient across the airway epithelium over this later period (Humbles *et al.*,1997). In the present investigation mRNA eotaxin expression appeared significant decrease in OVA- challenged group treated with GBE. This inhibition associated with the reduction of leukocytes migration and eosinophils infiltration in the BALF. Tumor necrosis factor alpha a protein manufactured by white blood cells to stimulate and activate the immune system in response to infection. Overproduction of this compound can lead to disease where the immune systems acts against healthy tissues. Some treatments for these diseases utilize drugs that bind and inactivate TNF- α , thereby reducing unhealthy inflammation (Berry *et al.*, 2007).As well as, tumor necrosis alpha (TNF- α) is a pro-inflammatory cytokine that has been implicated in the modulation of inflammation in various diseases , including asthma(Babu *et al.*,2004). The data obtained showed significant increase in TNF- α in OVA-challenged group compared to control group, which is in the same line with Babu *et al.* (2004). Moreover, the treatment with GBE suppressed TNF- α in the BALF of OVA-challenged group, which may be attributed to the binding of GBE to TNF- α receptor (Berry *et al.*, 2007) as a result, inactivate TNF- α reducing inflammation.

Ginkgo biloba has been used as an herb in traditional Chinese medicine for thousands of years. (Chu *et al.*,2011). The present results showed that GBE has a suppressing effect on airway inflammation by inhibiting the activity of cytokines and eotaxin, consequently inflammatory cell migration. That is in the line of our results which showed inhibitory effect on asthmatic lung by suppressing the levels of cytokines, eotaxin and cell infiltration.

Conclusion

The present results suggest that the cytokines levels, in addition to, mouse eotaxin reflect the intensity of eosinophilic airway inflammation as well as the disease activity, and may be useful as an inflammatory marker in asthma. Moreover, *Ginkgo biloba* leaf powder extract significantly decreased these inflammatory mediators and may serve as an alternative drug for patients with airway hyper-responsiveness.

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