

Effects of Inhaled L-Arginine Administration in A Murine Model of Acute Asthma

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ABSTRACT

Increased arginase activity in the airways decreases L-arginine and causes deficiency of bronchodilating and anti-inflammatory nitric oxide (NO) in asthma. As, it is suggested that L-arginine may have therapeutic potential in asthma treatment, we aimed to investigate the effects of inhaled L-arginine on oxygen saturation (SaO₂) and airway histology in a murine model of acute asthma.

Twenty eight BALB/c mice were divided into four groups; I, II, III and IV (control). All groups except the control were sensitized and challenged with ovalbumin. After establishment of acute asthma attack by methacholine administration, the mice were treated with inhaled L-arginine (Group I), saline (Group II) and budesonide (Group III), respectively. SaO₂ was measured by pulse oximeter just before and 5 min after methacholine. A third measurement of SaO₂ was also obtained 15 min after drug administration in these study groups. Inflammation in the lung tissues of the sacrificed animals were scored to determine the effects of the study drugs. The number of eosinophils in bronchoalveolar lavage (BAL) was determined.

The results indicated that inflammatory scores significantly improved in groups receiving study drugs when compared with placebo and L-arginine was similar in decreasing scores when compared with budesonide. SaO₂ had a tendency to increase after L-arginine administration after acute asthma attack and this increase was statistically significant ($p=0.043$). Eosinophilia in BAL significantly reduced in group receiving L-arginine when compared with placebo ($p<0.05$).

Thus in this study we demonstrated that L-arginine improved SaO₂ and inflammatory scores in an acute model of asthma.

Keywords: Acute asthma; Inflammation; L-arginine; Murine model; SaO₂

INTRODUCTION

Bronchial asthma is characterized by airway inflammation, epithelial damage, airway

hyperresponsiveness (AHR) and airway remodelling. Nitric oxide (NO) is involved in a variety of biological processes in lung and it has a controversial role in asthma.¹ Endogenous formation of NO is thought to

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have a role in smooth muscle relaxation, bronchodilation and neurotransmission beside inflammation.² L-arginine which is the donor of NO, importantly contributes to the development and progression of asthma. L-arginine metabolism is significantly altered in asthma due to increased expression of inducible NOS (iNOS) and also due to arginase enzyme which reduces L-arginine bioavailability by consuming endogenous L-arginine.^{3,4} Decreased bioavailability of L-arginine to eNOS causes bronchoconstriction since it is known that eNOS-derived NO results in bronchodilation through the cGMP pathway. Furthermore, iNOS-derived NO results in epithelial damage, infiltration of inflammatory cells, stimulation of TH2-mediated response, mucus hypersecretion, increased vascular permeability and airway hyperresponsiveness by formation of peroxynitrite rather than NO itself.^{5,6} Increased formation of peroxynitrite is known to be the result of decreased bioavailability of L-arginine to NOS. Increasing the L-arginine concentration in macrophages is found to stimulate NO production and inhibits the formation of peroxynitrite.^{7,8}

In this study, we aimed to investigate the effects of inhaled L-arginine on acute asthma and airway inflammation in a murine model of asthma and found that L-arginine administration may improve acute asthma attack and reduce airway injury.

MATERIALS AND METHODS

Animals

Conventionally raised, 6- to 8-week-old, 28 male BALB/c mice weighing 18 to 20 g were used in the experiment. The animals were fed a commercial diet ad libitum and housed in air conditioned facility on a 12-hour light/12-hour dark cycle. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. The Institutional Animal Care and Use Committee granted approval for the study.

Sensitization and Inhalation Exposure

Mice were sensitized by an intraperitoneal (ip) injection of ovalbumin (10 µg/0.1 ml, 2 weeks apart, ie on day 0 and day 14) consisting of a chicken egg albumin (ovalbumin, grade V, ≥98 pure, Sigma, St. Louis, MO) with alum adjuvant as described by Temelkovski et al.⁹ Mice in study groups I, II and III

were then challenged with an aerosol of 2.5% ovalbumin in saline for 30 minutes per day on 3 days of the week for 8 weeks beginning from the 21st day of the study. The mice in control group (group IV) received normal saline with alum (ip) on days 0 and 14 of the experiment and aerosolized saline without alum for 30 minutes per day on 3 days of the week for 8 weeks beginning from the 21st day of the study. Exposures were carried out in a whole body inhalation exposure system. Temperature and relative humidity were maintained at 20 C to 25 C and 40% to 60%, respectively. A solution of 2.5% ovalbumin in normal saline was aerosolized by a sidestream jet nebuliser which was injected into the chamber. The aerosol generated by this nebuliser comprised >80% particles with a diameter of <4 µm. Particle concentration was maintained in the range of 10 to 20 mg/mm^{3,10}.

Methacholine Administration and Study Drugs

On the last day of the nebulized ovalbumin administration, mice with chronic asthma in study groups I, II and III were given three doses of methacholine (at 6.25, 12.5 and 25 mg/ml concentrations) for 3 min by the same system used for administration of aerosolized ovalbumin.¹¹ The time interval between the subsequent doses of methacholine was 1 hour. SaO₂ was measured by pulse oximeter just before (0 min) and 5 min after 25 mg/ml concentration of methacholine. After that, mice in group I received nebulized L-arginine 1 M (174 mg/ml),¹² group II received saline, group III received nebulized budesonide 0.5mg/2 ml¹³ once. A third measurement of SaO₂ was also obtained 15 min after drug administration in the groups receiving L-arginine and placebo. Data about budesonide group was planned to be used in scoring of inflammation as budesonide is accepted as a gold standard treatment in asthma. L-Arginine was obtained from Sigma-Aldrich (St. Louis, MO). Schematic presentation of the study is presented in figure 1.

Bronchoalveolar Lavage

Bronchoalveolar lavage fluid (BAL) was immediately collected from euthanized mice by instillation and recovery of 1 ml 0.99 % NaCl through the tracheal cannula. BAL was centrifugated at 3000 X g for 10 min and supernatant was removed. Histological smear samples were prepared. A

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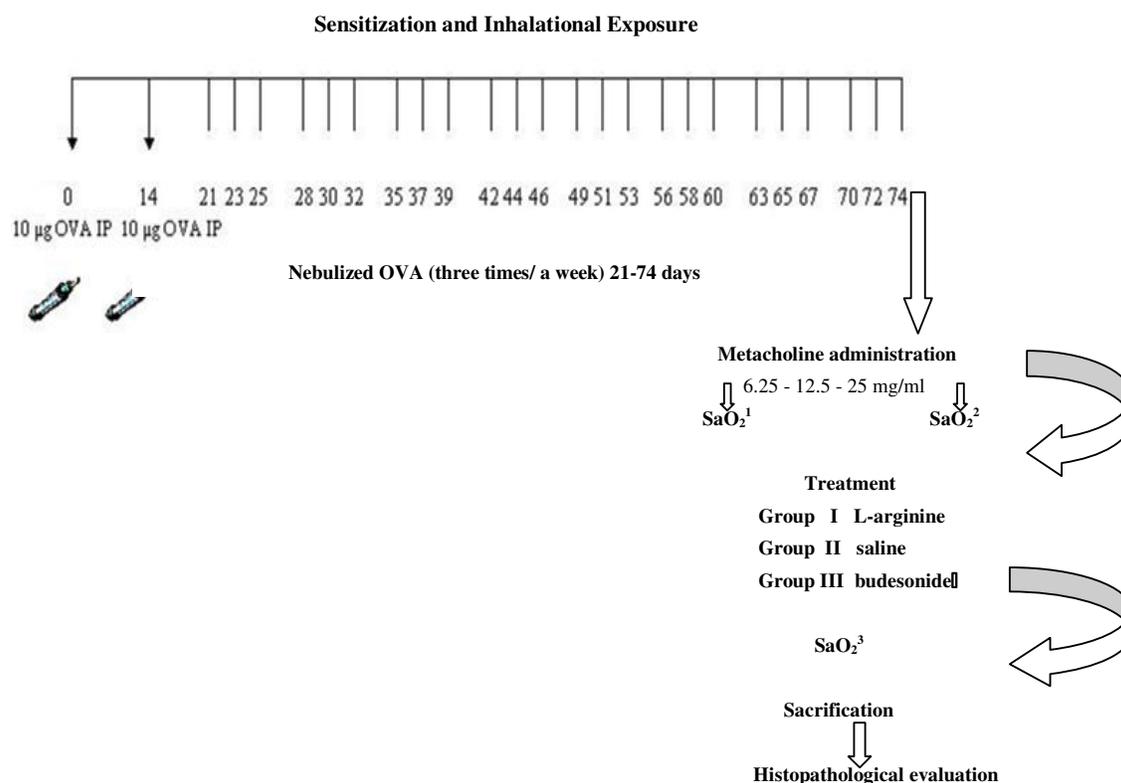


Figure 1. Schematic presentation for murine model of airway inflammation and study drugs

¹ SaO₂ measured before administration of metacholine,

² SaO₂ measured 5 minutes after administration of 25 mg/ml of metacholine,

³ SaO₂ measured 15 minutes after administration of L-arginine and placebo.

▮ Budesonide group was mainly created for histopathological evaluation and determination of interleukin levels.

differential cell count was performed using May-Grünwald Giemsa staining and cells were classified as eosinophils, neutrophils and lymphocytes on the basis of morphologic criteria and staining characteristics. Differential cell counts were performed in a blinded fashion by counting at least 200 cells per slide under light microscopy.

Histologic Evaluation

Examinations were conducted in a blind fashion by two of the investigators. Tissue specimens were obtained from the mid zone of the left lung of mice. Samples were fixed in 10% formalin for light microscopic evaluation. Some tissue samples of 1 to 2 mm³ taken from adjacent regions were stocked in 2% glutaraldehyde for electron microscopic evaluation. After fixation, samples were embedded in paraffin for

light microscopic evaluation and serial sections of 5-µm thickness were taken. After choosing the first section randomly, 10 sections in each mouse were selected by skipping over 10 sections and proceeded to staining process. For light microscopic evaluation, 3 different staining processes were used. The first 10 samples were stained with hematoxylin and eosin (H&E). In these samples general tissue features were examined and thicknesses of epithelium and subepithelial smooth muscle layers of the medium and small airways were measured. In order to evaluate the thicknesses of epithelium and subepithelial smooth muscle layers, measurements were performed from 4 points of each airway at levels of 3, 6, 9, and 12 o'clock. Considering that each section contained 2 to 3 airways, nearly 20 or more airways were evaluated in each mouse.

Photomicrographs were taken by JVC TK-890-E camera (Japan), which was adapted on Olympus BH-2 RFCA model microscope (Olympus Optical, Tokyo, Japan). The histological analysis was carried out with UTHSCSA Image Tool for Windows Version 3.00 software.

The consecutive 10 sections were stained with Toluidine Blue and the other 10 sections with periodic acid–Shiff (PAS). Photomicrographs were taken randomly from 5 fields of each section which were stained with Toluidine Blue. For mast cell enumeration, a standard transparent counting frame representing an area of 16400 μm^2 was used manually and 8 fields in each photograph were examined for each mouse. Goblet cells stained with PAS were enumerated in 10 sections of each mouse. In each section, randomly selected 3 to 5 airways were photographed. Circumferences of all airways were measured and goblet cell numbers in these areas were recorded. For standardization, goblet cell numbers in 100 μm were analyzed by division of total goblet cell number to the total length of airway circumferences and multiplying the result by one hundred.

Tissues were embedded in EPON after follow-up process of electron microscopic evaluation. Airways were marked from the semithin sections by light microscope. Ultrathin sections were obtained and stained with uranyl acetate and lead citrate. Libra 120 Carl Zeiss electron microscope (Oberkochen, Germany) was used for this evaluation. In each mouse, 5 to 7 ultrathin sections were taken from each 2 blocks and epithelium of the airway, the surrounding structures, and the intercellular connections were

evaluated.

In each mouse, 8 to 10 areas were photographed by Trondle (2048 \times 2048 pixel) digital camera, which was attached to the electron microscope. Thicknesses of the basement membrane of the respiratory epithelium were measured from 20 points that were at equal distances to each other and the data were recorded.

The degree of bronchial inflammation was also evaluated semi-quantitatively using scores of 0-3 to indicate no, mild, moderate and severe inflammation. Distribution and intensity of the following parameters were noted: 1) infiltration of inflammatory cells and fibrin from vessels into the mucosal and submucosal area of the bronchus and peribronchial interstitium, 2) bronchoconstriction (epithelial shedding or undulation of the nuclei of bronchial epithelial cells) 3) increase in number of goblet cells and 4) hypertrophy and thickening of the smooth muscle cell layer. A score of 0 indicated normal histology and a score of 3 indicated the greatest degree of alteration from normal.¹¹

Statistical analysis

SPSS 15.0 package program was used in the statistical analysis. Data were presented as mean \pm standard deviation (SD) in each group. Wilcoxon's signed rank test was used for the comparison of SaO₂ before and after metacholine and L-arginine/placebo administration. Histopathological scores were analyzed by Kruskal-Wallis and then Mann-Whitney test for dual comparisons. $P < 0.05$ was considered statistically significant.

Table 1. SaO₂ changes after metacholine and drug administration

SaO ₂ (%)	L-Arginine	Placebo
0. minute	93.14 \pm 4.87	85.28 \pm 6.82
5 minute after 25 mg/ml metacholine	78.71 \pm 15.15 \neq	84.42 \pm 20.37 \neq
15 minute after drug/placebo	97.0 \pm 3.69*	86.5 \pm 13.58 ¥

\neq Decrease in SaO₂ was not significant after 25 mg/ml of metacholine in both groups. * Significant increase in SaO₂ is present after administration of L-arginine ($p < 0.05$), ¥ Non-significant increase in SaO₂ is present after administration of placebo ($p > 0.05$)

Table 2. Comparison of pathological scoring[†] in study groups

Groups	Inflammation	Bronchoconstriction	Goblet cells	Subepithelial smooth muscle thickness
Control	0.28 \pm 0.18*	0.14 \pm 0.14*	0.0 \pm 0.0*	0.14 \pm 0.14*
Placebo	2.71 \pm 0.18	2.71 \pm 0.18	2.85 \pm 0.14	2.71 \pm 0.18
L-arginine	1.57 \pm 0.20* ¥	1.28 \pm 0.18* ¥	1.28 \pm 0.18* ¥	1.28 \pm 0.18* ¥
Budesonide	1.42 \pm 0.20*	1.28 \pm 0.18 *	1.28 \pm 0.18*	1.28 \pm 0.18*

[†] Scores of 0-3 to indicate no, mild, moderate and severe inflammation. * $p < 0.05$ when compared with placebo, ¥ $p > 0.05$ when compared with

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budesonide group

RESULTS

Response to Metacholine and Study Drugs

SaO₂ measurements before and after 25 mg/ml concentration of metacholine and after L-arginine and placebo administration are shown in Table 1. SaO₂ was found to decrease after metacholine but this decrease was not statistically significant. On the other hand, SaO₂ had a tendency to increase after L-arginine administration and this increase was statistically significant ($p=0.043$).

Histopathological Changes

The mean scores with respect to bronchial inflammation (infiltration of inflammatory cells and fibrin from vessels into the mucosal and submucosal area of the bronchus and peribronchial interstitium) were 1.57 ± 0.2 , 1.42 ± 0.20 , 2.71 ± 0.18 and 0.28 ± 0.18 in L-arginine, budesonide, placebo and control group, respectively. L-arginine significantly decreased inflammatory score when compared with placebo ($p<0.05$). The group treated with L-arginine showed similar scores with the group treated with budesonide ($p>0.05$) (Table 2).

The mean scores with respect to bronchoconstriction were 1.28 ± 0.18 , 1.28 ± 0.18 , 2.71 ± 0.18 , 0.14 ± 0.14 in L-arginine, budesonide, placebo and control groups, respectively. L-arginine significantly decreased scores of bronchoconstriction compared to placebo ($p<0.05$).

Scorings of goblet cells were significantly low in L-arginine and budesonide groups when compared with placebo ($p<0.05$) (Table 2). Scoring related to hypertrophy and thickening of smooth muscle cell layer were 1.28 ± 0.18 , 1.28 ± 0.18 , 2.85 ± 0.14 , 0.0 ± 0.0 in L-arginine, budesonide, placebo and control group, respectively.

Measurements regarding to subepithelial smooth muscle thickness, epithelial thickness, number of goblet and mast cells and thickness of basal membrane are presented in Table 3. All parameters were significantly improved in L-arginine and budesonide groups ($p<0.05$) (Table 3, Figure 2)

The number of eosinophils in BAL were $1.0\pm0.24\%$, $49\pm1.70\%$ and $22.2\pm1.01\%$ in control, placebo and L-arginine groups, respectively. Eosinophilia significantly reduced in group receiving L-arginine when compared with placebo ($p<0.05$).

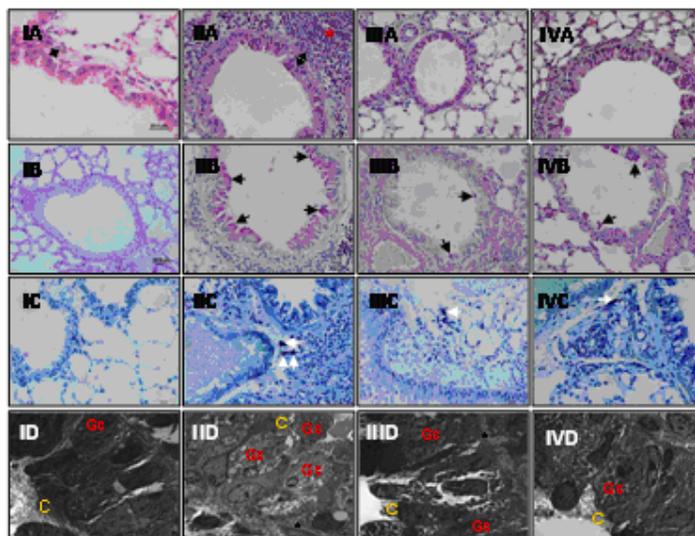


Figure 2. Light and electron microscopic findings of study groups. Figure 2. I; control (n:7), 2. II; placebo (n:7), 2. III; L-Arginine (n:7), 2. IV; budesonide (n:7) A; H&E, B; PAS, C; Toluidine staining. (A) Arrow with two heads; subepithelial smooth muscle thickening, (*) peribronchial mononuclear cell infiltration. (B) arrows; goblet cells (C) arrows; mast cells. (D) Electron microscopic views. Increased goblet cells in placebo group (Gc), basal membrane thickening(*). C; cilia.

Table 3. Histological comparison of the study groups

	Placebo (mean±SD)	Control	L-arginine	Budesonide
Subepithelial smooth muscle thickness (μm)	11.00 \pm 0.44	3.59 \pm 0.27*	4.49 \pm 0.24 *¥	8.16 \pm 0.37*
Epithelial thickness (μm)	23.61 \pm 1.17	10.99 \pm 0.42*	17.62 \pm 1.21*¥	18.92 \pm 1.44*
Number of goblet cells/100 (μm)	3.49 \pm 0.62	0.45 \pm 0.08 *	1.18 \pm 0.31*¥	1.49 \pm 0.38*
Number of mast cells /16400 (μm^2)	2.64 \pm 0.27	0.78 \pm 0.20*	0.98 \pm 0.27*¥	0.99 \pm 0.17*
Basal membrane thickness (μm)	677.0 \pm 18.87	366.7 \pm 8.48*	555.0 \pm 12.83*¥	533.7 \pm 19.36*

* $p < 0.05$ compared with placebo, ¥ $p > 0.05$ compared with budesonide

DISCUSSION

L-arginine has been evaluated in experimental models of asthma in various studies but effects of L-arginine on acute model of asthma was firstly investigated in our study. Using a histologically well established murine model of asthma, we demonstrated that L-arginine was effective in reversing abnormal histological findings of asthma and it had benefits in acute asthma setting.

Bioavailability of L-arginine is known to be reduced in asthma and it has been suggested that exogenous administration of L-arginine may increase the bronchodilating effect by increasing NO.¹⁴ L-arginine has been used as a potential therapy for asthma in various studies and different routes were used for administration of L-arginine.¹⁵ In the previous studies, oral and inhaled routes were used in humans while oral, inhaled and intraperitoneal routes were used in animals.^{4,16-19} Administration of oral L-arginine increased IL-5 expression and eosinophilia in bronchoalveolar lavage fluid in a murine model of asthma developed by Takano et al.¹⁸ In another study by Qadi-Nassar et al.,¹⁹ ip L-arginine was used and this treatment also increased eosinophils and neutrophils in BAL and increased airway responsiveness to inhaled metacholine. Inhaled L-arginine was used in two studies involving healthy and asthmatic subjects and effects of L-arginine on exhaled NO and also FEV₁ in the former were studied.^{16,17} The former study suggested that L-arginine increased the formation of endogenous NO and increase in NO had a negative correlation with FEV₁.¹⁶ The latter study ignored the rise in exhaled NO and suggested that the rise in FeNO had no clinical significance.¹⁷ In another study using a guinea pig model of asthma which used perfused tracheal preparations ex vivo, it was demonstrated that inhaled L-arginine reversed airway

hyperresponsiveness.⁴ The different results in all these studies can be explained because of the different species used, methodology of the studies and the dosage of L-arginine. Mabalirajan et al.³ stated that low doses of L-arginine may not be sufficient to increase the substrate availability of eNOS and high dose of L-arginine administered orally in their study resulted reduction of airway inflammation and airway hyperresponsiveness by increasing eNOS expression and downregulating both iNOS and arginase. We chose to use inhaled L-arginine administration in order to reach higher concentrations in airways rapidly and bypass the systemic effects of other routes. Our study was mainly based on oxygen saturation levels and histological outcome of L-arginine treatment in acute asthma setting. We noted improvement in oxygenisation and inflammaory scores of the study groups receiving L-arginine when compared to placebo.

We could not perform body plethysmography in order to determine the changes in pulmonary function tests during the study which may be regarded as a limitation of our study. We measured oxygenisation as a major outcome variable of acute asthma and histologically determined bronchoconstriction as a secondary outcome. Oxygenisation status was previously used in experimental animal studies of influenza and interstitial lung disease both of which suggest that pulse oximetry can be used as a predictor of lung pathology in mice.^{20,21} Our experiment design was based on our previous study about acute asthma in which the model was successfully established for the first time in the literature.¹¹

In conclusion, the current study showed that administration of inhaled L-arginine is effective in restoring oxygenisation status and improving histopathological changes including bronchoconstriction in a murine model of acute asthma. Thus, further

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animal and human studies are desirable to investigate the role of inhaled L-arginine in the development of better therapies for asthma.

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