

Changes in FeNO, d-ROMs, and BH₄ by Intravenous L-Arginine in Children and Its Putative Role in Asthma Treatment

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Purpose: Pteridines are metabolites of tetrahydrobiopterin (BH₄), being coenzymes for nitric oxide synthase (NOS). No study has clarified the relationship among pteridines and NOS, fractional exhaled nitric oxide (FeNO) generated by pteridines, and reactive oxygen species. In this study, we administered arginine, a precursor of NO, and confirmed changes in the levels of pteridines, FeNO, and reactive oxygen species and their relationship to clarify the pathogenesis of airway inflammation in which oxidative stress is involved, such as bronchial asthma.

Patients and Methods: This is a prospective, randomized open-label study. Children, aged 2 to 15 years, who were scheduled for growth hormone stimulation tests and were able to undergo a respiratory function test were recruited. They were randomly divided into two groups: arginine-administered and control groups. In the former, L-arginine hydrochloride was intravenously administered. After administration, the levels of diacron-reactive oxygen metabolites (d-ROMs), serum pteridines, serum amino acids, and fractional exhaled NO (FeNO) were measured.

Results: We analyzed 15 children aged 4 to 14 years. In the arginine-administered group, there was an increase in the FeNO level and a decrease in the d-ROMs level, reaching a peak 30 min after administration, compared with the control group. In addition, there was a decrease in the serum biopterin level and an increase in the d-ROMs level, reaching peak 60 min after administration.

Conclusion: The administration of L-arginine increased the NO level and decreased the d-ROMs level. Due to this, biopterin may be consumed and decreased, leading to an increase in the d-ROMs level. As a reduction in reactive oxygen species leads to the relief of inflammation, arginine and biopterin may be useful for inhibiting inflammation.

Keywords: asthma, biomarker, biopterin, child, FeNO, d-ROMs

Introduction

Concerning bronchial asthma, it was reported that the fractional exhaled nitric oxide (FeNO) level was increased in asthma patients with eosinophilic inflammation of the airway.^{1,2} This parameter is particularly useful as a marker of the severity of asthma or for monitoring; therefore, it is used in clinical practice.^{3–6}

Nitric oxide (NO) is generated in a synthetic pathway from L-arginine to L-citrulline in the presence of tetrahydrobiopterin (BH₄) as a coenzyme and nitric oxide synthase (NOS) as a catalyst.^{7,8} NOS has been isolated into three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), which affects FeNO production.⁹ BH₄ acts as an essential coenzyme for all subtypes of NOS. In the respiratory system, iNOS produces NO and increases FeNO concentrations above normal in the presence of airway inflammation. NO has a variety of roles in the body, especially in the respiratory tract, NO relaxes airway smooth muscle and acts as an airway protective agent. In addition, NO works together with active oxygen to form free radicals, which can cause inflammation and damage to the lungs and airways. In other words, NO has both airway protective and inflammatory effects.¹⁰

Previous studies indicated that oral administration or inhalation of L-arginine transiently increased the body's (including expired air) level of NO.^{11,12} When BH₄ is insufficient, which is a coenzyme for the synthetic pathway, NOS produces reactive oxygen species instead of NO (Figure 1).¹³ There is evidence that allergic diseases are caused by excessively produced reactive oxygen species/oxidative stress. Previous studies have also reported that oxidative stress played an important role in asthma.^{14–17} Interventional studies with arginine involving patients with asthma have also been conducted.^{18,19} However, no study has clarified changes in BH₄ or reactive oxygen species associated with the arginine-administration-related promotion of NO synthesis.

We hypothesized that administration of L-arginine activated NO synthesis pathway, BH₄ (coenzyme) insufficiency may increase the level of oxidative stress, such as reactive oxygen species, but not NO production, contributing to the essential condition of bronchial asthma and chronic airway inflammation. To test this hypothesis, we intravenously administered L-arginine to healthy children to clarify the pathogenesis of airway inflammation.

Patients and Methods

Subjects

We recruited patients in whom pediatric endocrinologists considered a growth hormone stimulation test necessary for the diagnosis of short stature in Osaka City University of Medicine Hospital (current name: Osaka Metropolitan University Hospital) between February 2017 and February 2018. Inclusion criteria were children, aged 2 to 15 years, in whom it is possible to perform a respiratory function test for measuring FeNO. The exclusion criteria included patients with a history of allergic reactions to arginine and those in whom inhaled/oral/intravenous steroids had been used within 1 month before an arginine loading test. The clinical criteria for exclusion included known allergies to L-arginine and the use of corticosteroid 1 month before growth hormone stimulation tests.

This study was approved by the Institutional Review Boards at Osaka City University of Medicine (No. 3634). Written informed consent was obtained from all parents, and children were asked for their assent. This study was conducted in accordance with the Declaration of Helsinki.

Study Design

This is a prospective, randomized open-label study. Before the study, all subjects underwent an objective questionnaire about their history of allergy, use of anti-allergic drugs, and family history of smoking, blood tests, and nasal eosinophil test. During the study days, all subjects fasted in the morning. The subjects were randomly allocated to two groups. One group (arginine-administered group) received L-arginine hydrochloride on the first day, and the other

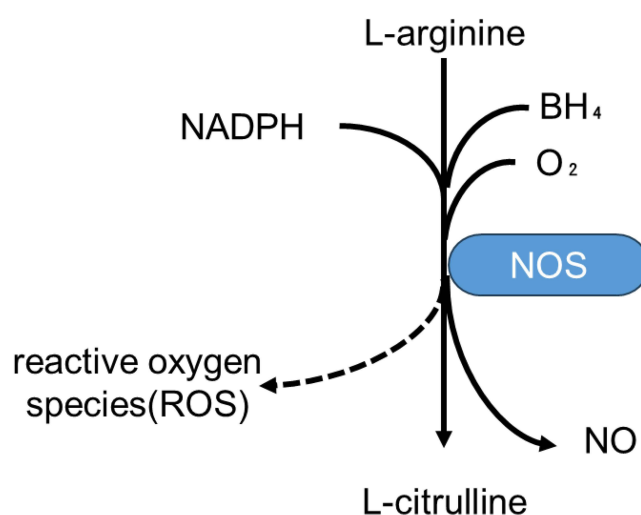


Figure 1 The metabolic pathway of NO generation. NO is generated from L-arginine to L-citrulline by the nitric oxide synthase (NOS) in some cofactors, biopterin, O₂, nicotinamide-adenine dinucleotide phosphate (NADPH). Reduced levels of BH₄ or L-arginine lead to uncoupling of reduced NADPH oxidation and NO synthesis, resulting in the generation of reactive oxygen species.

group (control group) received 10mg/kg L-Dopa (Dopaston Powder, Ohara Pharmaceutical, Japan) orally on the first day to assess the response of growth hormone secretion. L-arginine hydrochloride (AY Pharmaceuticals; Tokyo, Japan) was administered as 500 mg/kg (max 30 g).²⁰ Infusion started at 9:00 am, which was designated time 0. Diacron-reactive oxygen metabolites (d-ROMs), serum pteridines, serum amino acid (AA), and fractional exhaled nitric oxide (FeNO) were measured at 0, 30, 60, 90, and 120 min. Vital signs were measured at fixed time before and after administration.

Diacron-Reactive Oxygen Metabolites

The levels of diacron reactive oxygen metabolite (d-ROMs) were measured using a free radical analyzer system (FRAS4; Wismerll Company, Tokyo, Japan) according to the manufacturer's protocol. The d-ROMs test reflects the amount of serum organic hydroperoxides (ROOH) that reflects the free radicals from which they are formed. Briefly, 20 µL of serum was mixed with an acid buffer solution of pH 4.8 in a cuvette, which was then supplemented with the chromogen colorless. Chromogen was oxidized by free radicals and converted to a radical cation with a pink color.

Pteridine Analysis

Pteridines are metabolites of BH₄. Of these, biopterin reflects BH₄ in vivo; therefore, the biopterin level was measured. Measurement of serum pteridines was performed by high performance liquid chromatography (HPLC, SHIMADZU LC-10) as previously described.²¹ Briefly, 100 µL of serum samples were oxidized with iodine, separated by high-performance liquid chromatography, and quantified with a fluorescence detector to measure biopterin levels.

Fractional Exhaled Nitric Oxide

The FeNO level was measured using NIOX VERO devices (Aerocrine AB; Stockholm, Sweden) according to American Thoracic Society guidelines.²² In short, the subjects were required to inhale deeply via a mouthpiece, then exhale with a constant flow (0.05 L/s) for 6 s.

Serum Amino Acid Analysis

The serum amino acid concentrations were measured using a Hitachi L-8800 Amino Acid Analyzer (Tokyo, Japan), as previously described.²³ Briefly, 100 µL of samples were hydrolyzed by hydrochloric acid and then loaded on a Hitachi L-8800 amino acid analyzer for amino acid analysis.

Statistical Analysis

To examine the influence of arginine loading tests on d-ROMs, we conducted multivariable linear regression analysis using the amount of change in d-ROMs from a baseline (0 min) until each measurement point (30 min, 60 min, 90 min, and 120 min) as an objective variable, the presence or absence of arginine loading and measurement point as qualitative variables, and their interaction term as an explanatory variable. In this model, the influence of the baseline d-ROMs value (0 min), age, sex, and date of treatment (order of treatment) was adjusted. To handle the data repeated from each patient, the data correlation was analyzed using the Huber-White robust estimation method. Furthermore, FeNO was similarly investigated. For analysis, we used R version 4.0.3 software.

Results

Demographic and Characteristics of Patients

We recruited 15 patients. The subjects' characteristics are shown in Table 1. The ages ranged from 4 to 14 years (mean ages in the arginine-administered and control groups: 8.11 (SD 3.30) and 8.67 years (SD 3.88), respectively). No subject had a history of asthma or a history of respiratory tract infection within 4 weeks before the start of this study. The T-IgE levels in the arginine-administered and control groups were 656.96 and 427.57, respectively. In the former, there was an increase in the blood concentration of arginine, reaching a peak 30 min after administration (Figure 2, Table 2).

Table 1 Demographic Characteristics of the Subjects

	With Arginine (n=9)	Without Arginine (n=6)	p value
Age, years, mean±SD	8.11±3.30	8.67±3.88	0.77
Male sex, no.(%)	5 (56)	3 (50)	
Total IgE (IU/l)	656.96±940.99	427.57±947.17	0.65
Blood eosinophils (/mL)	350.44±225.66	170.33±171.30	0.12
Serum arginine (μmol/l)	0.94±1.72	0.28±0.16	0.40
Serum bipterin (nmol/L)	2.21±0.44	2.07±0.62	0.62
d-ROMs (U.CARR)	355.56±73.93	300.50±45.28	0.13
FeNO (ppb)	23.11±15.11	14.00±15.94	0.28
Doctor diagnosed BA, no (%)	0 (0)	0 (0)	
Doctor diagnosed AR, no (%)	3 (33)	0 (0)	
Doctor diagnosed AD, no (%)	1 (11)	1 (11)	
Parental smoking, no (%)	7 (78)	0	

Comparison of Changes in the FeNO Level Between the Two Groups

Figure 3 shows changes in the FeNO level before drug administration and 30, 60, 90, and 120 min after administration in the arginine-administered and control groups. In the former, the FeNO level significantly increased compared with the latter from 30 min after administration. There was a peak 30 min after administration (7.679; 95% CI, 4.137–11.222; $p=0.000$). In the arginine-administered group, there remained a significant increase in the FeNO level compared with the control group even 120 min after administration (5.888; 95% CI, 2.933–8.843; $p=0.000$). The FeNO level reached a peak 30 min after administration (Table 3).

Comparison of Changes in the d-ROMs Level Between the Two Groups

Figure 4 shows changes in the d-ROMs level before drug administration and 30, 60, 90, and 120 min after administration in the arginine-administered and control groups. In the former, the d-ROMs level decreased at maximum 30 min after administration (−36.717; 95% CI, −58.139– −15.294; $p=0.001$), but gradually increased thereafter. There was no significant difference 90 min after administration (Table 4).

Comparison of Changes in the Bipterin Level Between the Two Groups

Figure 5 shows changes in bipterin levels before drug administration and 30, 60, 90, and 120 min after administration in the arginine-administered and control groups. In the former, there was a decrease in the bipterin level 60 min after

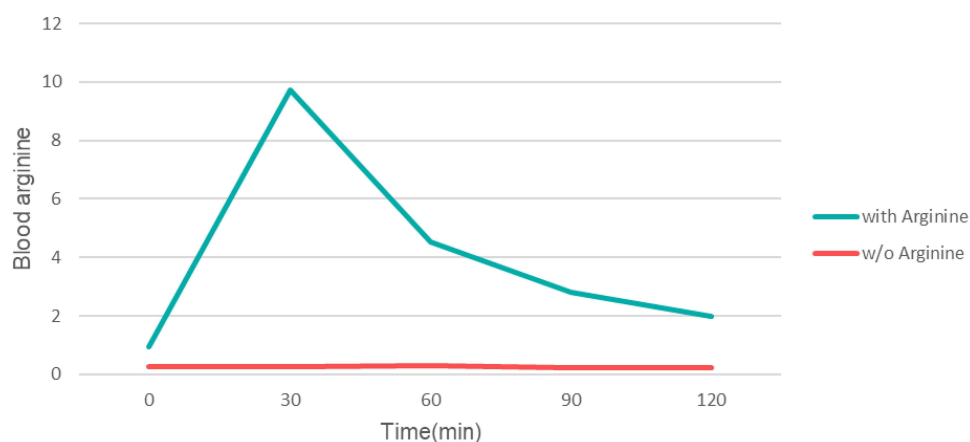
**Figure 2** Blood arginine concentration.

Table 2 Blood Arginine Concentration

Time	With Arginine	Without Arginine
0 min	0.94±1.71	0.28±0.16
30 min	9.72±4.28	0.28±0.17
60 min	4.54±1.94	0.30±0.19
90 min	2.81±1.05	0.23±0.15
120 min	1.97±0.86	0.22±0.15

administration. In the latter, its level increased from 30 min after administration. There was a significant difference compared with the arginine-administered group between 30 and 90 min after administration (30 min: -1.041 ; 95% CI, -1.445 - -0.637 ; $p=0.000$) (Table 5).

Discussion

In this study, L-arginine administration increased the level of FeNO, which was likely to promote bronchodilation and reduce inflammation, and decreased the level of d-ROMs, which was likely to reduce inflammatory activity in the body including the lungs. These findings indicate that L-arginine administration may contribute to the improvement of asthmatic symptoms in children. Previously, Knatitonov et al reported that oral L-arginine administration to healthy subjects increased the expired air NO level.¹¹ Sapienza et al indicated that inhalation of L-arginine in healthy subjects and patients with asthma increased the expired air NO level.¹⁸ Similarly, this study also showed that intravenous L-arginine administration increased the expired air NO level. This NO level promptly increased after L-arginine administration, but slightly decreased thereafter. L-arginine was intravenously administered in this study, and thus the expired air NO level more promptly increased in comparison with previous studies.

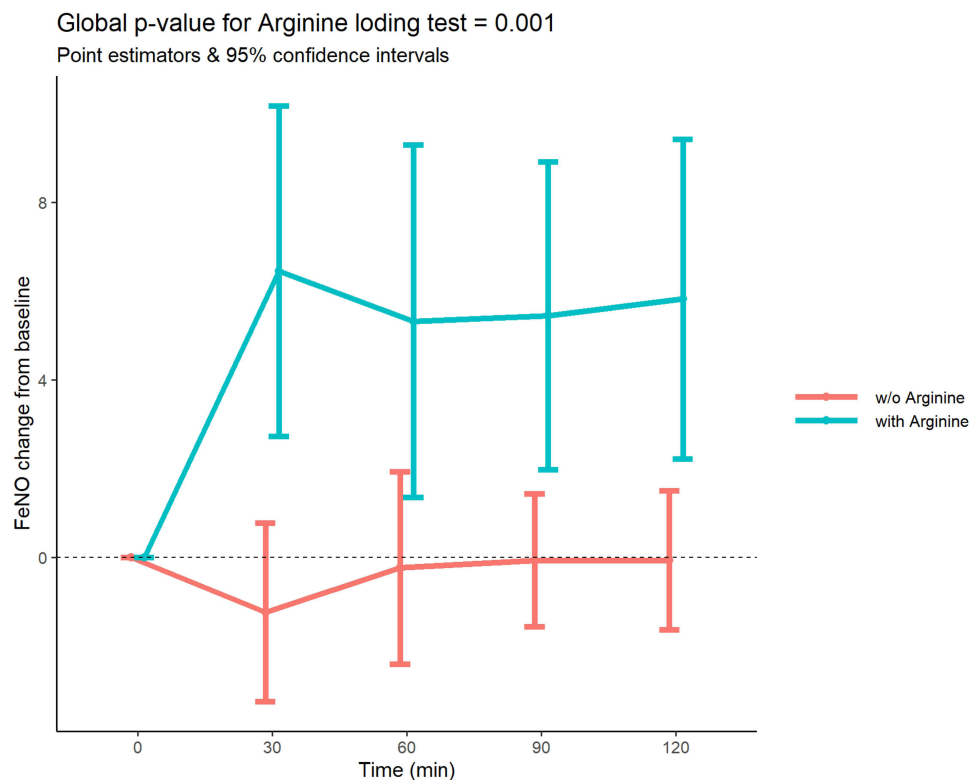
**Figure 3** Comparison of changes in FeNO between with arginine and without arginine.

Table 3 The Effect of Arginine Loading Test for FeNO

Time	Effect	Lower 95% CI	Upper 95% CI	p value
30 min	7.679	4.137	11.222	0.000
60 min	5.554	1.948	9.161	0.003
90 min	5.513	2.61	8.416	0.000
120 min	5.888	2.933	8.843	0.000

NO has both anti-inflammatory and inflammatory effects in the airways and may play a role in amplifying the inflammatory response in the airways in asthmatic patients with higher FeNO levels.²⁴ In asthmatic airway inflammation, FeNO levels increase through iNOS, particularly due to the involvement of type 2 inflammation.²² This study also revealed that intravenous L-arginine administration decreases the levels of d-ROMs as well as increases FeNO. As a reduction in reactive oxygen species leads to relief from inflammation, having the potential for therapeutic effects. Ayyildiz et al demonstrated that inhalation of L-arginine improved arterial oxygen saturation (SaO₂) and eosinophil count in bronchoalveolar lavage (BAL) using a mouse asthma attack model.²⁵ Their study suggested that an extrinsic L-arginine administration-related increase in the NO level enhances bronchodilator effects. Another study suggested that L-arginine administration influences arginine metabolism in a mouse asthma model, contributing to the improvement of asthmatic symptoms.²⁶ A recent clinical trial of a citrulline supplement involving obese asthmatics showed that citrulline increased the FeNO level, adequately improving the forced expiratory volume in 1 s (FEV1).¹⁹ Based on these reports and the present study, it is possible that the increase in FeNO and decrease in dROMs after L-arginine administration in the present study may lead to improvement of asthma symptoms and respiratory function.

In contrast, there was a significant decrease in the biopterin level in the L-arginine-administered group in comparison with the non-L-arginine-administered group from 60 min after administration in this study. In the urea cycle, L-arginine is used as a material and biopterin is used as a cofactor for NOS to synthesize L-citrulline and simultaneously synthesize

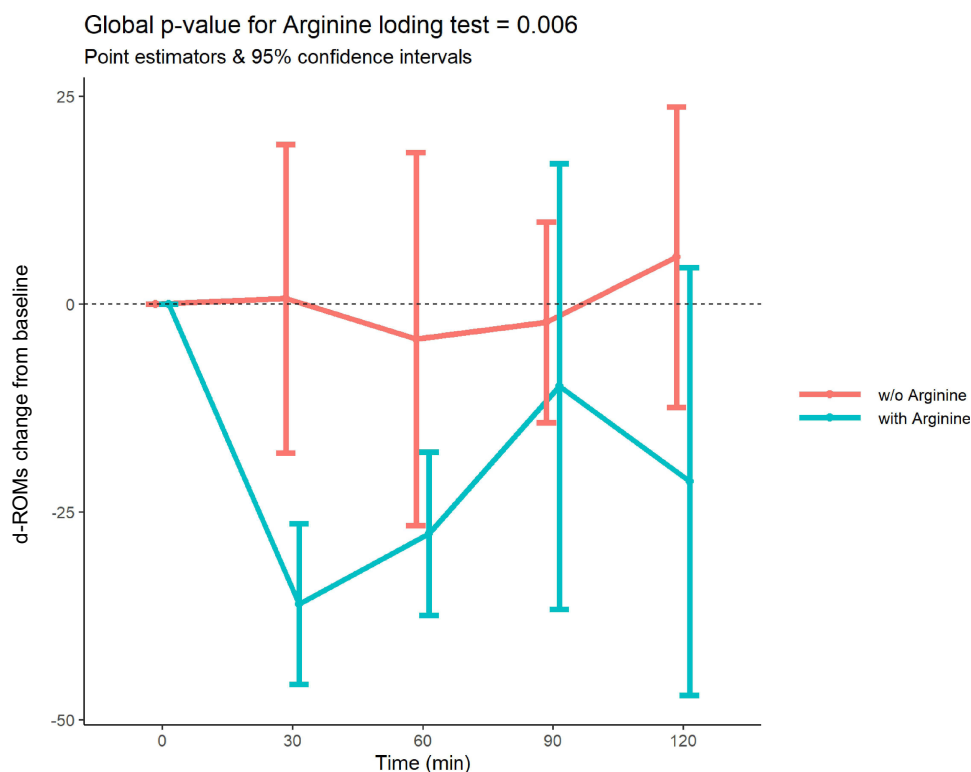
**Figure 4** Comparison of changes in d-ROMs between with arginine and without arginine.

Table 4 The Effect of Arginine Loading Test for d-ROMs

Time	Effect	Lower 95% CI	Upper 95% CI	p value
30 min	-36.717	-58.139	-15.294	0.001
60 min	-23.446	-44.784	-2.107	0.032
90 min	-7.696	-39.176	23.784	0.625
120 min	-26.967	-54.216	0.283	0.052

NO. When the urea cycle is activated by L-arginine administration, biopterin is consumed and when temporarily insufficient, ROS are produced in place of NO (Figure 1).¹³ This study suggested that the biopterin level may decrease from 60 min after administration due to biopterin consumption by the urea cycle and the levels of d-ROMs may have increased. Recently, an interventional clinical study involving arginine administration to severe asthmatic patients was conducted to improve the route of arginine metabolism. However, there was no therapeutic effect of arginine on asthma.²⁴ Takeda et al reported a negative correlation between FeNO and pteridine in children with asthma.²⁷ Kasuga et al revealed that asthmatic children tended to have lower neopterin and biopterin levels.²⁸ Based on these reports, we assumed that biopterin was quickly insufficient by arginine administration alone, and the synthetic pathway shifted to the generation of oxidative stress instead of NO because serum biopterin level was low in patients with more severe asthma. This suggested that the administration of biopterin in addition to arginine inhibits the synthesis of reactive oxygen species, being effective for asthma treatment. Currently, for clinical research on BH₄ supplementation, clinical trials involving BH₄ administration for chronic diseases including respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis, are being conducted. An improvement in endothelial function in patients with these diseases was reported.^{29,30} In the future, BH₄ administration may be useful as a new treatment option.

The first limitation of this study was that the subjects were patients in whom pediatric endocrinologists considered an arginine loading test necessary, considering invasiveness related to arginine administration to healthy subjects. For this

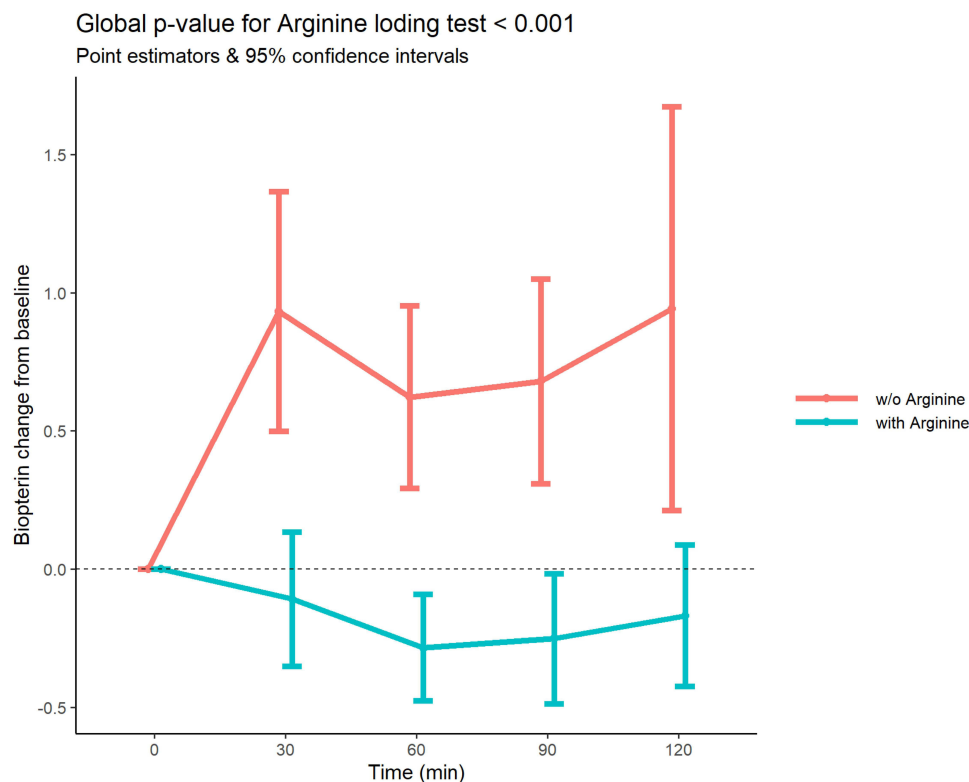
**Figure 5** Comparison of changes in biopterin between with arginine and without arginine.

Table 5 The Effect of Arginine Loading Test for Biopterin

Time	Effect	Lower 95% CI	Upper 95% CI	p value
30 min	−1.041	−1.445	−0.637	0.000
60 min	−0.906	−1.28	−0.533	0.000
90 min	−0.93	−1.288	−0.572	0.000
120 min	−1.111	−1.859	−0.362	0.005

reason, the number of subjects was small. As the second limitation, the observation time of 120 min was short enough to confirm that the effects of arginine administration had normalized. As the third limitation, a base at the same dose as arginine was not administered in the control group from ethical aspects. In the arginine-administered group, drug administration may have reduced blood concentration. As the fourth limitation, the biopterin level increased in the control group. The structure of L-Dopa resembles that of phenylalanine (GenomeNet Database: COMPOUND: C00355), and L-Dopa administration may have promoted GTP cyclohydrolase I (GTPCH I) activity, increasing the BH₄ level.^{31,32} However, no study has reported an L-Dopa-administration-related promotion of GTPCH1 activity or an increase in the BH₄ level. In this study, it was difficult to predict this phenomenon before administration as a loading test.

Conclusion

In conclusion, there was a decrease in the d-ROMs level after L-arginine administration, and there was a significant decrease in the level of biopterin as an alternative for BH₄. Subsequently, there was an increase in the d-ROMs level. As a reduction in reactive oxygen species leads to the relief of inflammation, arginine and biopterin may be useful as a treatment method for inhibiting inflammation. Further research is essential to clarify that the administration of arginine and biopterin inhibits the synthesis of reactive oxygen species, being as effective as asthma treatment.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure

Prof. Dr. Ayumi Shintani reports grants and/or personal fees from Kyowa Kirin, AstraZeneca K.K., Asahi Kasei Pharma Corporation, Chugai Pharmaceutical Co, Daiichi Sankyo Co, Merck Biopharma, Pfizer Japan Inc, Takeda Pharmaceutical Co, Shionogi Pharma, and Janssen Pharmaceutical K.K, outside the submitted work. The authors report no other conflicts of interest in this work.

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