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Effect of growth hormone therapy on nitric oxide formation in cystic fibrosis patients

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Running title**: Growth hormone in cystic fibrosis**

Abstract

Rationale: **Airway nitric oxide production is decreased in cystic fibrosis. As growth hormone therapy has been shown to increase nitric oxide production in growth hormone deficiency, it may also affect nitric oxide production in patients with cystic fibrosis.**

Objectives: **To study the effect of growth hormone therapy on systemic and airway nitric oxide formation in patients with cystic fibrosis.**

Measurements: **Nitric oxide metabolites in serum and urine, amino acid concentrations in serum and sputum, as well as exhaled nitric oxide was measured in children with cystic fibrosis before, during and after one year of treatment with human growth hormone.**

Results: **Nitric oxide metabolite concentrations increased significantly in serum and urine during the treatment period. Serum amino acids concentrations including L-arginine, the substrate for nitric oxide synthases also increased during treatment. The systemic bio-availability of L-arginine for nitric oxide synthases, expressed as ratio of L-arginine/L-ornithine+lysine, remained unchanged. In contrast, L-arginine concentrations in sputum decreased significantly during growth hormone treatment, as did exhaled nitric oxide levels.**

Conclusions: **Treatment with growth hormone in children with cystic fibrosis decreases exhaled nitric oxide by reducing the concentration of L-arginine in the airways.**

Key words: hormones, inflammatory marker, pulmonary function, L-arginine

Introduction

Cystic fibrosis (CF) is an inherited disease caused by mutations in the CFTR gene. The clinical course of CF is variable but classically characterized by malabsorption, dystrophy secondary to pancreatic insufficiency, as well as chronic airway infection and inflammation leading to progressive loss of pulmonary function and ultimately respiratory failure [1]. CF airways are deficient for nitric oxide (NO). The mechanisms behind low NO formation in CF airways are incompletely understood but may include deficiency of L-arginine, the substrate of NO synthases (NOS) and decreased expression of NOS [2-5].

There is increasing evidence that low NO concentrations are functionally relevant in CF. Positive correlations between exhaled NO (FE_{NO}) and pulmonary function have been repeatedly observed [6-7]. In addition, gene variants in neuronal NOS (*NOS1*), that are associated with low FE_{NO}, seem to predispose to a more rapid decline in pulmonary function in CF children [8]. An increased risk for the colonization of CF airways with *P. aeruginosa* was also found to be related to *NOS1* and *NOS3* gene variants associated with low FE_{NO} [9-10].

L-arginine supplementation, on the other hand exhibits anti-inflammatory effects as shown in a rat model of chronic Pseudomonas infection [11] and, when added to the airways, has dilatatory effects on airway smooth muscle in CFTR-deficient mice [12]. L-arginine may also have protective effects on pulmonary oxidative stress and antioxidant defenses [13]. In CF patients L-arginine supplementation resulted in an increase in FE_{NO} when given

intravenously or orally [14-15], and a single inhalation of nebulized L-arginine was shown to increase FE_{NO} and FEV_1 in CF patients [16].

NO formation can be augmented by increasing the availability of L-arginine but also by increasing NOS expression [17]. Endocrine factors such as estrogen, IGF-1 and growth hormone have also been found to modify the L-arginine/NO pathway [18, 19]. Growth hormone is capable of transcriptional activation of NOS [20, 21] and may also affect substrate availability for NOS as it enhances L-arginine uptake by the small intestine [22, 23]. Growth hormone deficiency results in decreased systemic NO formation and replacement of growth hormone in deficient patients leads to increased NO, recovered urinary nitrate and cGMP production, normalization of elevated vascular peripheral resistance and improved cardiac output [24, 25].

Therapy with human growth hormone (hGH) has been shown to deploy beneficial effects for CF patients including enhanced growth, weight gain and bone mineralization [26-28]. Since GH replacement resulted in increased NO metabolism in GH-deficient patients, we hypothesized that GH therapy in CF patients would also result in increased NO formation. We therefore studied the effect of hGH therapy on L-arginine metabolism and NO formation in CF patients undergoing hGH therapy as part of a multicenter trial to assess the effect of hGH therapy in CF [26].

Materials and Methods

Study design

Patients included in this study participated in a randomized placebo-controlled, multicenter study on the effects of hGH therapy in CF (Pharmacia trial 307 MET-9002-026) [26]. Patients were treated with recombinant hGH by daily subcutaneous injection with 0.039 mg/kg body weight per day (~0.11 IU/kg body weight per day), 0.07 mg/kg body weight per day (~0.21 IU/kg body weight per day) or placebo. The study was conducted as a 24-week double-blind study with a 24-week open-label treatment period. Patients were assigned to one of three treatment arms for 24 weeks. After the end of the double-blind treatment period, patients on GH therapy were maintained on their current GH dosage for an additional 24 weeks and patients in the placebo group were randomly assigned to either the low or the high GH-dosage treatment regimen. Subjects had to have an established diagnosis of CF, dystrophy defined by a BMI <10th and/or weight <3rd percentile despite high caloric intake (>120% RDA), and a bone age of 8-18 years. Exclusion criteria were acute pulmonary exacerbation, diabetes, and liver cirrhosis. Patients were studied before initiation of hGH treatment, every three months during therapy, as well as six and twelve months after the treatment period.

Study population

Fifteen CF patients from a single centre (Essen), that all participated in the multicenter study, were included in this sub-study. Eleven patients were treated with hGH for 12 months; six patients with low dose and five with high dose of hGH. Four patients, that were assigned to the placebo group and therefore did not receive hGH for 12 months, were not included in the analysis. Mean (range) age in the eleven patients was 14 [12-18] years, FEV_1 53 (28-90) % predicted, and BMI SDS was -1.7 (-0.9 to -3.7); which was not different from

the total study population [26]. NO metabolites (NOx) were measured in both serum and urine before, during (three, six, nine, and 12 months), and twelve months after hGH therapy. Serum amino acids were measured before and during (six and nine months) hGH therapy. Sputum was primarily collected for microbiology. Amino acid concentrations in sputum were measured in five GH treated patients able to provide additional samples during the blinded phase of the study (before, and after three and six months) as well as six months after hGH therapy. Exhaled NO was measured in all patients before, during (three, six, 9 and 12 months) and six months after hGH treatment. FeNO after 12 months of hGH therapy was only measured in four patients and therefore these data were not analyzed.

Serum and sputum sample processing

Blood was drawn by venous puncture, immediately centrifuged and serum stored at -20°C until analyzed. Spontaneously expectorated sputum was used primarily for microbiology. Additional samples were diluted with buffer 1:1, vortexed for 2 min followed by 10 min of centrifugation at 1000g. Clear supernatant was used for amino acid measurements, respectively.

NOx measurements

Total NO metabolites (nitrate, nitrite and nitrosothiols) were measured after conversion to NO by using vanadium (III) chloride in hydrochloric acid at 90°C with an NO analyzer (NOA 280 and purge vessel, Sievers, Boulder, CO, USA) [29].

Amino acid measurements

Amino acids were determined by ion exchange chromatography on an amino acid analyzer LC 3000 (Eppendorf, Hamburg, Germany) according to the manufacturer's specifications. Serum and sputum samples were deproteinized before analysis.

Exhaled NO measurement

A chemiluminescence analyzer (NOA 280, Sievers, Boulder, CO, USA) was used to measure FE_{NO}. Single breath on-line measurements for the assessment of lower airway NO were performed at a constant expiratory flow of 50 ml x min-1, ($FE_{NO\ 50}$) in accordance with published ERS/ATS standards [30-31]. The NO analyzer was calibrated before each study with 0 and 185-ppb NO calibration gas (Linde AG, Unterschleissheim, Germany). The mean of three end-expiratory NO concentrations within a variation of 15% was calculated for each subject.

Statistics

Data are shown as median ± interquartile range. Comparisons of time courses in NO metabolite (NOx) and amino acid concentrations, as well as FE_{NO} were done by Kruskal-Wallis one-way analysis of variance (ANOVA). Comparison of L-arginine bioavailability index (L-arginine / L-ornithine+lysine) in serum vs. sputum was done by Wilcoxon test.

Results

There was no difference between the low and high hGH treatment groups in any of the parameters analyzed. Serum NO metabolite (NOx) concentrations increased significantly

(p=0.027, ANOVA) during the treatment period and returned to baseline values after hGH was discontinued (Figure 1). Similarly, urine NOx concentrations also increased significantly (p=0.029, ANOVA) during treatment and returned to baseline after hGH was discontinued (Figure 2).

Serum concentrations of 18 amino acids measured before and during hGH therapy are displayed in Table 1. With the exception of threonine, mean levels of all amino acid in serum significantly increased during hGH treatment. This included L-arginine and L-citrulline, the substrate and the product of NOS activity respectively, as well as L-ornithine, the product of arginase and proline, a downstream product of the arginase pathway and precursor of collagen formation (Table 1). The ratio of L-arginine / L-ornithine+lysine, which can be used as an index of bio-availability of L-arginine for NOS at a given L-arginine concentration [32], was 0.45 ± 0.08 (mean ± SD) in serum before treatment and remained unchanged during hGH treatment (p=0.347, ANOVA).

Mean exhaled NO (FE_{NO}) decreased significantly during the treatment period (p=0.005, ANOVA) and returned to baseline after hGH was discontinued (Figure 3).

L-arginine concentration in CF sputum was 15.5 ± 17.3 μmol/L at baseline and decreased significantly during hGH treatment (p=0.008, ANOVA) (Figure 4A). The L-arginine availability index at baseline was significantly lower in CF sputum than in serum (p< 0.001, Wilcoxon test). Individual changes in L-arginine bio-availability index in sputum are displayed in Figure 4B. Although this index decreased in individual patients, the mean

sputum L-arginine bio-availability index did not change significantly during hGH treatment (p=0.275, ANOVA).

There was no correlation between pulmonary function data and **any of the parameters analyzed.**

Discussion

In this study we report that treatment of CF patients with hGH resulted in increased serum amino acid concentrations including L-arginine, the substrate for NO synthases, as well as increased NO metabolite concentrations in both serum and urine. These findings suggest increased vascular or systemic formation of NO during hGH therapy in CF patients and parallel those in patients deficient for growth hormone, where hGH replacement therapy increased systemic NO formation [25]. In contrast, concentrations of L-arginine in sputum as well as exhaled NO levels decreased during hGH treatment, suggesting decreased substrate availability for NOS and decreased NO formation in airways of CF patients treated with hGH.

Increased NO production during hGH treatment, as evidenced by increased NOx in serum and urine, could result from effects of GH on the expression or activity of constitutive or inducible NOS isoforms. Experiments in cultured human endothelial cell had shown that GH resulted in a dose- and time-dependant increase in eNOS (NOS3) gene and protein expression [20]. In a study in cultured mesangial cells GH treatment was also found to result in a dose-dependent increase in iNOS (NOS2) transcript [21]. iNOS is of interest in CF since, despite the inflammatory nature of the disease, its expression is decreased in CF airway epithelial cells [3, 4]. The mechanisms resulting in decreased iNOS expression in CF are currently not understood, however even activation with pro-inflammatory cytokines, that result in profound iNOS activation in non-CF cells have limited to no effect on iNOS expression in CFTR deficient cells [5]. Translation of NOS2 mRNA can also be downregulated by low concentrations of L-arginine [33, 34].

Another plausible explanation for higher systemic NO metabolite concentrations in CF patients treated with hGH is increased enzymatic NO formation in response to higher substrate availability. L-arginine conversion by NOS is known to increase with higher extracellular L-arginine levels, although the intracellular concentrations of L-arginine are usually well above the *K*m for NOS. This phenomenon is referred to as the L-arginine paradox [35], and is thought to be responsible for the increase in FE_{NO} in healthy subjects but also CF patients supplemented with L-arginine [14, 15]. Thus, the observed increase in NO metabolite levels may be directly related to the circulating concentrations of L-arginine, as both L-arginine and NOx increased in serum.

While systemic L-arginine and NO metabolite production increased in CF patients during GH therapy, at the same time, NO in the exhaled air decreased. FE_{NO} is used as a surrogate marker for NO production in airway; thus low FE_{NO} would suggest decreased airway NO production. Alternatively, retention of NO in airway secretions and conversion to metabolites such as nitrite or nitrate could result in low FE_{NO} in the presence of unchanged or even increased enzymatic NO production. This mechanism had previously been suggested to be one contributing factor to low FE_{NO} in CF patients [36, 37]. In the present study, however, we found that treatment with hGH resulted in decreased substrate availability for NOS in CF airways. Although, due to the small sample size, these results need to be interpreted with caution, we found that not only absolute sputum L-arginine concentration but also the relative bioavailability of L-arginine for NOS decreased in CF sputum during therapy with hGH. The L-arginine bioavailability index (L-arginine / L-

ornithine+lysine) reflects availability of L-arginine for NOS at a given L-arginine concentration, as L-arginine, L-ornithine and lysine utilize the same transmembrane transport system, the cationic amino acids transporter (CAT)-2 [32] for cellular internalization. This index has recently been shown to be decreased in blood of patients with asthma, sickle cell disease and CF, and is thought to reflect substrate limitation for NOS-mediated smooth muscle relaxation in these conditions [2, 32, 38]. Even at baseline this index was significantly lower in sputum compared to serum in CF patients. This reduced L-arginine availability for CF airway cells may potentially affect the ability of upregulating NO as part of a defense mechanism against airway pathogens sensitive to NO-mediated killing, such as mucoid *P. aeruginosa* [39].

Chronic infection in CF results in neutrophil dominated inflammation and increased concentrations of inflammatory markers in the airways [1]. Activation of signal transducer and activator of transcription (STAT) 1 however, is reduced in CF and the IFNγ signaling pathway, which involves STAT1 and leads to NOS2 gene induction in airway epithelial cells, is defective in CF [40]. Thus inhibitory effects of GH on these pathways may further decrease the expression of NOS2 in CF. Indeed, GH was found to suppress the synergistic induction of iNOS by the cytokines IFNγ and TNFα in a rat insulin-secreting cell line (INS-1 cells). This effect was thought to be mediated through inhibition of IFNγ-activated STAT1 by GH [41]. In contrast, iNOS enzyme activity in liver tissue from calves undergoing LPS challenges was not affected by pretreatment with GH. However, GH treatment before LPS resulted in an increase in cNOS activity, the phosphorylation of NOS3, plasma NOx levels, and liver protein nitration, in GH treated compared to non-GH

treated animals. Of interest, the progressive increase in tissue CAT-2 mRNA after LPS was significantly reduced with GH treatment in this model [42], potentially contributing to substrate limitation for NOS. Although not yet studied in CF, these studies may suggest that GH therapy in CF patients could have significant effects on NO formation by affecting the expression of NOS2 and by reducing L-arginine availability for NOS.

Low airway NO concentration may not only reflect decreased production but may also result from the reaction of NO with reactive oxygen species (ROS). Activated NOS can release reactive oxygen species such as O_2^- and H_2O_2 at non-saturating L-arginine levels [43, 44], and NOS can generate NO and O_2^- at the same time at low-saturating L-arginine levels [45]. These products may react to generate peroxynitrite, and subsequently result in tyrosine nitration. Nitrotyrosine had been previously found to be increased in CF lung tissue, sputum and exhaled breath condensate [46]. Previous studies had suggested that exogenous GH primes human and rat neutrophils, monocytes and macrophages for a respiratory burst and an accompanying increase in superoxide (O_2^-) production [47, 48]. In contrast, NO release by cultured endothelial cells treated with GH was only increased at GH concentrations high enough to also result in a significant reduction of ROS formation. At lower concentrations, the expression of NOS3 was increased but NO was metabolized by reacting with ROS [20]. Therefore, the effect of GH on NO production may be dose dependent and higher doses than used in the present study may potentially have different effects on pulmonary NO production.

In summary, the results of our study suggest that treatment with hGH results in decreased airway NO formation in patients with CF. Further studies are needed to clarify whether this observation is specific for CF and to specify the pathophysiological consequences of the observed changes in the L-arginine/nitric oxide metabolic pathway.

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Figure Legends

Figure 1: NO-metabolite (NOx) concentrations in serum before, during and after 12 months of hGH treatment in CF patients (n=11). Serum NOx increased during the treatment period and returned to baseline values after hGH was discontinued.

Figure 2: NO-metabolite (NOx) concentrations in urine before, during and after 12 months of hGH treatment in CF patients (n=11). Urine NOx concentrations increased during treatment and returned to baseline after hGH was discontinued.

Figure 3: Fractional exhaled nitric oxide (FE_{NO}) in CF patients before, during and after 12 months of hGH treatment (n=11). Mean FE_{NO} decreased during the treatment period and returned to baseline after hGH was discontinued.

Figure 4: L-arginine (A) and L-arginine bioavailability index (B) in sputum of CF patients before, during and after 12 months of hGH treatment (n=5). L-arginine concentrations

decreased during hGH treatment, while the mean L-arginine availability index did not change.

Table 1: Serum amino acid concentrations (in μmol/L) in 10 CF patients before as well as six and nine months after the initiation of therapy with hGH.

Amino acid	pre	6 months	9 months	p-value
L-arginine	105.6 (92.5-127.6)	110.8 (100.7-122.3)	138.4 (114.1-165.5)	0.027
L-ornithine	79.3 (54.5-116.9)	75.1 (63.2-94.9)	126.0 (89.5-135.5)	0.038
L-citrulline	24.5 (18.0-32.4)	22.7 (13.4-25.4)	37.5 (27.1-45.7)	0.006
Threonine	140.5 (102.8-205.3)	128.1 (100.9-167.4)	206.3 (133.8-235.5)	0.090
Serine	132.7 (112.8-171.5)	151.4 (137.6-171.4)	191.3 (168.2-222.3)	0.002
Proline	166.2 (125.9-242.4)	148.6 (115.7-194.6)	224.6 (178.1-254.8)	0.028
Taurine	137.6 (123.9-166.2)	172.3 (136.5-193.8)	175.8 (154.5-203.4)	0.032
Glycine	280.3 (235.7-403.9)	343.9 (312.3-410.7)	436.7 (369.7-485.6)	0.005
Alanine	387.8 (301.7-450.5)	415.4 (365.5-461.0)	469.6 (407.7-573.9)	0.042
Valine	161.1 (148.4-187.1)	165.2 (145.9-200.8)	237.0 (187.2-271.1)	0.006
Methionine	8.3 (3.6-11.6)	5.1 (3.6-8.7)	10.4 (7.5-16.3)	0.016
Isoleucine	47.1 (35.5-88.3)	42.9 (36.3-55.3)	83.0 (65.1-98.8)	0.008
Leucine	79.1 (56.4-107.6)	88.0 (77.8-109.1)	140.6 (114.3-181.5)	0.002
Tyrosine	43.7 (32.2-80.7)	44.4 (40.9-52.3)	67.2 (51.6-76.0)	0.048
Phenylalanine	54.7 (40.2-70.9)	51.8 (44.3-65.4)	77.5 (67.4-94.9)	0.013
Histidine	68.0 (57.8-87.9)	75.0 (66.6-86.3)	94.4 (83.8-109.4)	0.005
Tryptophan	45.3 (35.3-49.6)	40.3 (32.8-55.8)	56.3 (47.3-64.8)	0.031
Lysine	134.6 (125.5-205.3)	131.3 (118.3-161.5)	208.6 (151.1-230.9)	0.018

P-values given are for Kruskal-Wallis ANOVA.

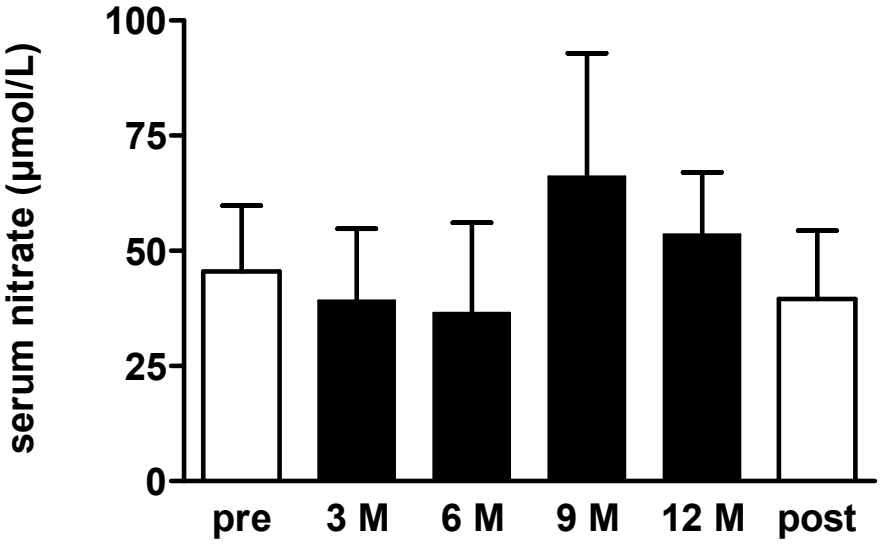

Figure 1

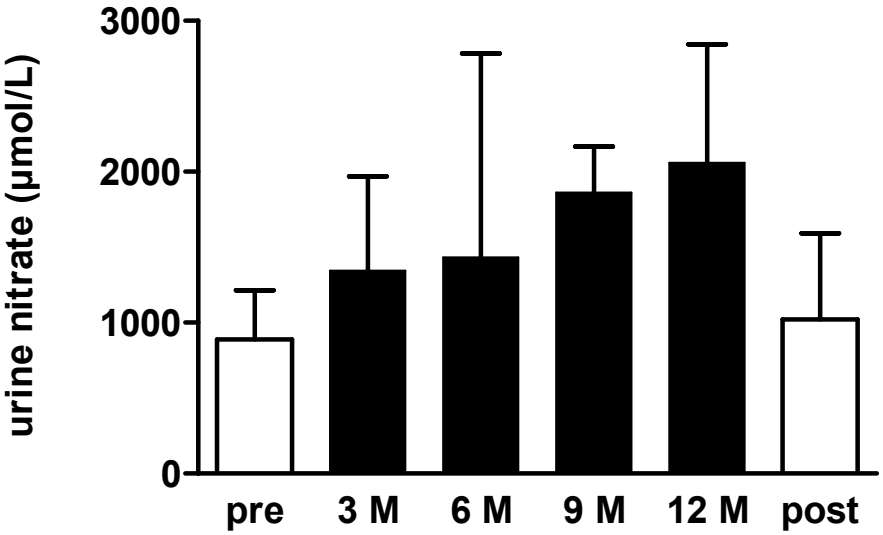

Figure 2

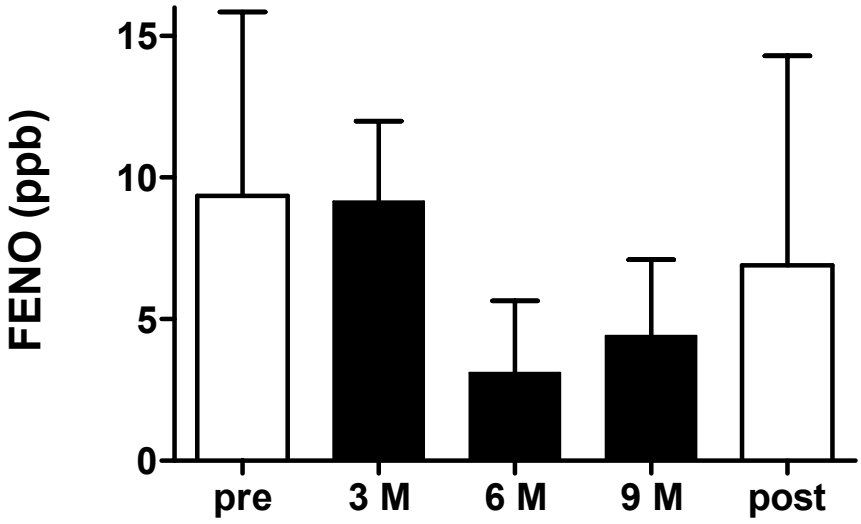

Figure 3

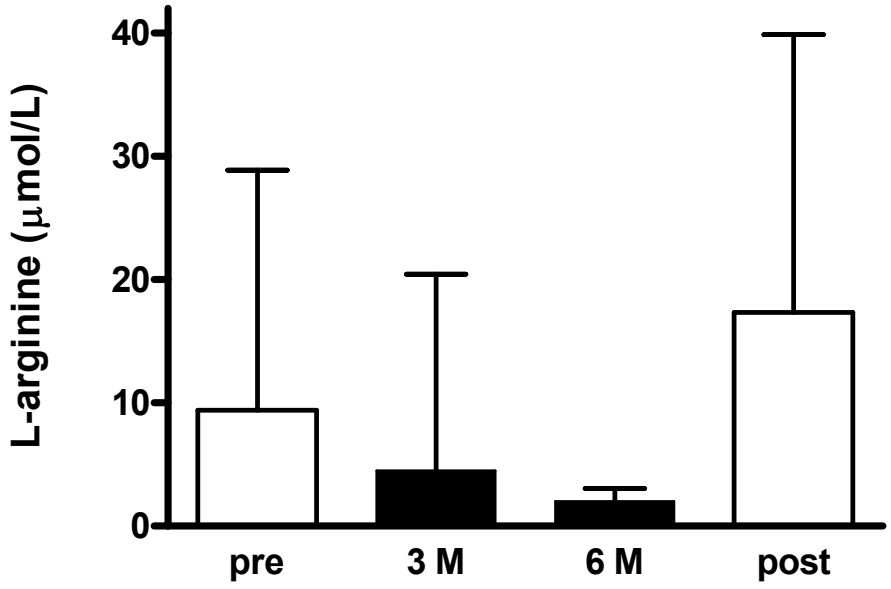

Figure 4 A

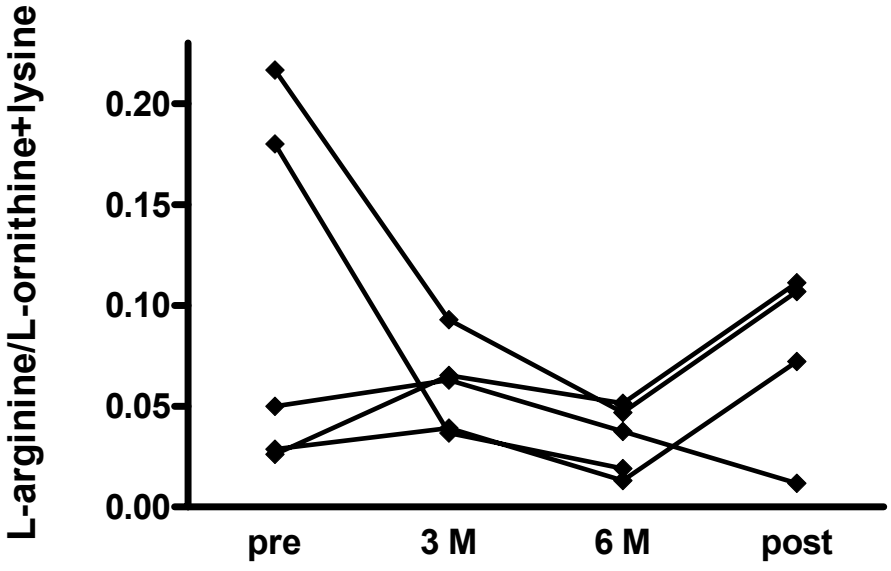

Figure 4 B