主論文

Pleiotropic effects of linagliptin monotherapy on levels of nitric oxide, nitric oxide synthase, and superoxide dismutase in hemodialysis patients with diabetes

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Running title: (Pleiotropic effects of linagliptin monotherapy)

Abstract

Background: Linagliptin is an anti-diabetic drug that is also the only bile-excreted dipeptidyl peptidase-4 (DPP-4) inhibitor. Malnutrition-inflammation-atherosclerosis syndrome is an important prognostic factor for hemodialysis patients, and we have previously reported the anti-inflammatory effects of linagliptin in hemodialysis patients with diabetes. Inflammation is known to accelerate oxidative stress, vasoconstriction and platelet aggregation. However, very few studies have investigated the pleiotropic effects of linagliptin treatment on inflammation in hemodialysis patients. In this study, we investigated these effects in a longer and more thorough follow-up of hemodialysis patients with diabetes.

Methods: We examined 20 hemodialysis patients with diabetes who were not receiving oral diabetes drugs or insulin therapy and exhibited inadequate glycemic control (glycated albumin levels, >20%). Linagliptin (5 mg) was administered daily, and we evaluated the patients' superoxide dismutase, 8-hydroxydeoxyguanosine, nitric oxide, nitric oxide synthase, and asymmetric dimethylarginine levels in serum at baseline and after 1, 3, and 6 months of treatment.

Results: After 6 months of treatment, superoxide dismutase levels had significantly decreased from 8.8 ± 0.5 U/mL to 7.0 ± 0.5 U/mL. Nitric oxide synthase levels were significantly increased at 3 and 6 months (maximum, 94.2 ± 13.2 μg/mL; baseline, 31.6 ± 5.5 μg/mL). After 3 months of treatment, nitric

oxide levels had significantly increased from 64.5 ± 6.6 μmol/L to 104 ± 15.4 μmol/L, and remained significantly elevated at 6 months. Asymmetric dimethylarginine and 8-hydroxydeoxyguanosine levels did not change during the 6-month treatment course, and no patients exhibited hypoglycemia or other significant adverse effects.

Conclusion: Linagliptin treatment significantly changed various markers of inflammation, which are related to atherosclerosis in malnutrition-inflammation-atherosclerosis syndrome. Therefore, linagliptin monotherapy has pleiotropic effects on inflammation in hemodialysis patients with diabetes, and may improve their prognosis.

Key words: inflammation, anti-oxidation, hemodialysis, linagliptin, atherosclerosis

INTRODUCTION

Hemodialysis (HD) patients have a high prevalence of protein-energy malnutrition, inflammation and atherosclerotic cardiovascular disease. Since these three pathophysiological conditions occur concomitantly in HD patients by the actions of pro-inflammatory cytokines, they have been referred to as malnutrition-inflammation-atherosclerosis syndrome (MIA syndrome). MIA syndrome is an important prognostic factor for HD patients (1). In addition, diabetes mellitus can induce end-stage renal disease and promote the inflammation of MIA syndrome. Although insulin injections are central to the treatment of HD patients, eyesight failure due to diabetic retinopathy and aging-associated dementia can contraindicate multiple daily insulin injections (2). Moreover, among HD patients, many oral anti-diabetic drugs induce critical side effects, such as hypoglycemia and lactic acidosis. Therefore, new oral anti-diabetic drugs with fewer side effects are needed for HD patients. In this context, dipeptidyl peptidase-4 (DPP-4) inhibitors are well tolerated, have a lower incidence of hypoglycemia, and provide a good safety profile.

Linagliptin is an anti-diabetic drug that is also the only bile-excreted DPP-4 inhibitor, and does not require a reduced dose in HD patients with diabetes. In addition, we have previously reported the anti-inflammatory effects of linagliptin in HD patients with diabetes (3). After initiating linagliptin treatment, the levels of prostaglandin E2, interleukin 6, and glycated albumin (GA) decreased significantly, whereas levels of glucagon-like peptide-1 (GLP-1)

increased significantly. Interestingly, inflammation is known to accelerate oxidative stress, vasoconstriction and platelet aggregation. As these factors are related to the atherosclerosis in MIA syndrome, linagliptin therapy reduces the risk of cardiovascular and cerebrovascular diseases (CCV) (4), which are related to systemic atherosclerosis and the related prognostic factors. Given the anti-inflammatory effects of linagliptin, this drug may suppress oxidative stress, vasoconstriction and platelet aggregation. However, there are very few studies that have investigated the pleiotropic effects of linagliptin.

The present study is an extension and a more thorough follow-up of our previous study (3). In the present study, we investigated the pleiotropic effects of linagliptin monotherapy treatment on inflammation in HD patients with diabetes.

METHODS

Patients

For the present study, we included 20 HD patients (16 men, 65.5 ± 2.8 years old) with diabetes and inadequate glycemic control (GA levels of >20%) who were also adhering to diet and exercise therapy. We selected GA to reflect glycemic control, as hemoglobin A1c (HbA1c, the more common parameter) is artificially reduced in HD patients (5-7). Among the 20 patients that we included, 4 had a history of insulin therapy, 8 were taking other oral anti-diabetic drugs, and 8 had been treated with both insulin and other oral

anti-diabetic drugs. However, their therapy had subsequently been discontinued before or after their initiation of maintenance dialysis therapy, with an average washout period of >2 years. Thus, at the start of the study, no patient was using any prescribed oral anti-diabetic drug or receiving insulin injections, and their glycemic control had become inadequate. In addition, no patients were taking non-steroidal anti-inflammatory drugs or allopurinol. During the treatment period, the patients received a once-daily oral dose of linagliptin (5 mg). The ethics committee of Saiyu Soka Hospital approved this study's design, and informed consent was obtained from each patient.

Efficacy

The following efficacy parameters were examined at baseline and after 1, 3, and 6 months of treatment: oxidative stress (superoxide dismutase [SOD] activity and high sensitivity 8-hydroxydeoxyguanosine [8-OHdG] levels) and vasodilatation and platelet aggregation dysfunction factors (nitric oxide [NO], nitric oxide synthase [NOS], and asymmetric dimethylarginine [ADMA] levels).

Safety assessments

The safety assessments involved monitoring for all adverse events, including hypoglycemic events, which were assessed via blood glucose measurements. To address possible hypoglycemic events, a 24-h treatment support system was

made available to all patients, which allowed them to receive immediate treatment if they experienced any symptoms of hypoglycemia. Moreover, participants underwent capillary glucose monitoring before commencing their HD treatment. Hematological and blood biochemical assessments, evalution of the participants' vital signs and physical condition were perfomed.

Blood samples

For the present study, we evaluated the same blood samples that were used in our previous study (3). These blood samples were taken from the arterial side of the arteriovenous fistula, before the start of HD treatment and 1–2 h before eating. Caution was exercised to prevent hemolysis of the samples, the plasma was obtained via centrifugation, and was subsequently stored at −70° C until the analysis.

Measurements of the parameters

The patients' SOD activity was measured using a batched-reagent set at a single laboratory (SRL Laboratory, Hachioji, Tokyo, Japan) via an improved nitrite method, which estimates the activity based on the decreasing rate of nitrite that is produced by hydroxylamine and superoxide anions. The patients' 8-OHdG levels were measured using the high-sensitivity 8-OHdG enzyme-linked immunosorbent assay (ELISA; Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd., Fukuroi, Shizuoka, Japan), which evaluates

8-OHdG as a product of oxidized DNA that is formed by hydroxyl radicals, singlet oxygen, and direct photodynamic action. The patients' NO levels were measured using the QuantiChrom™ Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA, USA), which measures NO production after the reduction of nitrate to nitrite via the improved Griess method. The patients' NOS activity was measured using the EnzyChrom™ Nitric Oxide Synthase Assay Kit (BioAssay Systems, Hayward, CA, USA), which uses a 2-step process, whereby NOS produces NO that is subsequently detected and quantified. The patients' ADMA levels were measured using reverse-phase high performance liquid chromatography with AccQ Tag amino acid analysis. This method uses 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (a derivatizing agent), which has a derivatized amino acid yield of 100% (8).

Statistical analysis

JMP statistical software (version 10; SAS Institute, Cary, NC, USA) was used for all statistical analyses, and the results were presented as mean ± standard error. Significance was tested using the paired t-test, and differences with a P-value of <0.05 were considered statistically significant.

RESULTS

Patient history

The baseline characteristics of the 20 patients are shown in Table 1. Six

patients were smokers, 9 were receiving antiplatelet drugs, 2 were receiving vasodilator drugs, 6 were receiving 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, and 3 patients were receiving angiotensin receptor blockers. Each patient had received treatment for type 2 diabetes prior to the start of maintenance dialysis therapy, with a mean treatment period of 10.4 ± 2.1 years. Dialysis treatment was conducted 3 times per week in 4-h sessions, with a mean treatment duration of 6.0 ± 1.9 years. During HD treatment, the mean blood flow was 201.0 ± 4.9 mL/min (range, 160–250 mL/min), the mean volume of the dialysate was 515.0 ± 10.9 mL/min (range, 500–700 mL/min), the mean dialysis time was 4.1 ± 0.1 h (range, 4–4.5 h), the mean membrane area was 1.8 ± 0.1 m^2 (range, 1.5–2.1 m^2), and the total glucose concentration in the dialysates was 100 mg/dL.

Effect of linagliptin on SOD, NO, and NOS levels

The patients' SOD levels decreased significantly from 8.8 ± 0.5 U/mL at baseline to 7.0 ± 0.5 U/mL at 6 months after starting linagliptin ($P < 0.05$) (Fig. 1). However, the patients' NO levels increased significantly from 64.5 ± 6.6 μmol/L at baseline to 104 ± 15.4 μmol/L at 3 months after starting linagliptin ($P < 0.05$) (Fig. 2). Similarly, their NOS levels increased significantly at 3 months after starting linagliptin ($P < 0.05$), and increased to a maximum value of 94.2 ± 13.2 μg/mL (vs. 31.6 ± 5.5 μg/mL at baseline) at 6 months (Fig. 3). There were no changes in the levels of ADMA and 8-OHdG (Table 2).

No adverse effects of linagliptin treatment

Hypoglycemia is a potential side effect of diabetes therapy, although a meta-analysis of clinical trial data revealed that only a small number of hypoglycemic events were associated with vildagliptin and sitagliptin treatment (other DPP-4 inhibitors) (9). However, over the 6-month course of the present study, no patients exhibited hypoglycemia or other significant adverse effects.

DISCUSSION

Inflammation is an important prognostic factor for HD patients, and the pleiotropic effects of inflammation are relevant when treating HD patients. However, few studies have examined the pleiotropic effects of linagliptin monotherapy on inflammation in HD patients with diabetes. Therefore, we conducted the present study to evaluate this issue, and found that linagliptin treatment significantly decreased the levels of SOD, while increasing the levels of NO and NOS.

We have previously reported the anti-inflammatory effects of linagliptin in HD patients with diabetes (3), and proposed 4 potential mechanisms for the anti-inflammatory effects of linagliptin: increased GLP-1, suppression of DPP-4 (CD26), an effect of the xanthine-related skeletal systems, or an anti-diabetic effect. In the present study, in addition to the anti-inflammatory effects of linagliptin, we observed a significant decrease in the levels of SOD (an

oxidative stress marker). In this context, increased levels of GLP-1 have both anti-oxidant and anti-inflammatory activities (10-12). In addition, DPP-4 is expressed as CD26 on the membranes of various cells, including leucocytes, where it acts as an inflammatory mediator, with functions in T-cell activation, DNA synthesis, cell proliferation, cytokine production, and signaling activation. Therefore, oxidative processes may be modulated by DPP-4 inhibition (13, 14). One clinical trial provided a single daily dose of linagliptin (5 mg) to patients with diabetes, and reported that DPP-4 was inhibited by >80% for 24 h after treatment (15). In addition, compared to other DPP-4 inhibitors, linagliptin exhibited stronger inhibitory activity and selectivity for DPP-4. Furthermore, among the 7 types of DPP-4 inhibitors, linagliptin is the only DPP-4 inhibitor with a xanthine-based skeletal structure, and the pharmacological mechanisms of xanthine-based skeleton structures' anti-oxidant and anti-inflammatory effects remain unclear (16). Thus, it is possible that this structure suppresses degradation of cyclic adenosine monophosphate (cAMP) via phosphodiesterase inhibition (thereby increasing intracellular cAMP concentration). In addition, hyperglycemia induces monocytes to release interleukin 6 (an inflammatory cytokine) via protein kinase C induction (17), and also stimulates pro-inflammatory cytokine production via c-Jun *N*-terminal kinase in monocytic THP-1 cells (18). We have confirmed the antidiabetic effects of linagliptin in the patients of this study previously (3). The levels of GA, the parameter of glycemic control, were significantly decreased after starting linagliptin therapy,

and there was very little difference in the effects of linagliptin therapy among their patients. Therefore, improved glycemic control may help prevent oxidative stress, although further studies among HD patients are needed to confirm the anti-oxidant effect of linagliptin.

In patients with type 2 diabetes, GLP-1 promotes the eNOS-mediated increase in vasodilatation, and prevents tumor necrosis factor α-induced expression of plasminogen activator inhibitor-1 and vascular cell adhesion molecule-1 in vascular endothelial cells (19, 20). However, few studies have examined the effect of linagliptin on vasodilatation and platelet aggregation dysfunction in HD patients with diabetes. In our previous study, GLP-1 levels increased 2.5-fold after linagliptin treatment (3), and NO (which promotes vasodilatation and platelet aggregation dysfunction) increased with increasing NOS levels in the present study. In addition, ADMA (an endogenous NOS inhibitor) is dependent on the GLP-1 receptor's signal (21), and the absence of any significant increase in ADMA levels (in the present study) might have contributed to the increased levels of NO that we observed.

Oxidative stress, vasodilatation, and suppression of platelet aggregation are also important factors that are related to atherosclerosis. Therefore, the anti-atherosclerotic effects of linagliptin treatment may be related to the decreased SOD levels and increased NO and NOS levels that we observed (22, 23). Peripheral arterial disease results from systemic atherosclerosis (24), and is an important prognostic factor for HD patients (25). In this context, the ankle

brachial pressure index (ABI) is particularly useful in functionally evaluating peripheral arterial disease, and the rate of ABI's decline predicts cardiovascular mortality in HD patients (26). Therefore, we have previously investigated the change in ABI after 2 years of linagliptin treatment in a cohort of 20 HD patients (the linagliptin group), and compared it to the change in ABI that we observed among 20 HD patients with diabetes who received once-daily doses of 6.25 mg alogliptin (the alogliptin group). Our unpublished results revealed that ABI levels decreased from 0.97 ± 0.03 to 0.92 ± 0.07 in the linagliptin group, compared to from 0.97 ± 0.05 to 0.83 ± 0.05 in the alogliptin group. In addition, significantly fewer patients who received linagliptin ($n = 6$) experienced a decrease in their ABI levels, compared to the patients who received alogliptin ($n = 14$, $P < 0.05$). This difference may suggest that linagliptin has stronger anti-atherosclerotic effects than alogliptin.

This study has several important limitations that should be considered when interpreting our results. First, the present study did not include a control group and only evaluated a small number of participants. However, as none of the patients were receiving diabetic drugs before or after the initiation of maintenance dialysis therapy, any improvement that we observed in the parameters was likely related to the linagliptin treatment. Second, we did not observe any change in the levels of 8-OHdG (an oxidative stress marker) during the linagliptin treatment, which does not indicate a whole-body anti-oxidant effect. However, as SOD is the enzyme that catalyzes the conversion of

superoxide into oxygen and hydrogen peroxide, the anti-oxidant effects of linagliptin may be related to this activity. Third, SOD is not the only marker for oxidative stress, and NO and NOS are not the only markers for vasodilation. Therefore, we hope to evaluate the effects of linagliptin on various other markers for oxidative stress and vasodilation. Moreover, we measured enzyme activities of SOD and NOS in this study but not expression levels of their isozymes. Since each enzyme has three types of isozymes, it is important to address this issue in the future studies.

In conclusion, among HD patients with diabetes, linagliptin has pleiotropic effects on inflammation, and those effects may improve these patients' prognosis. Therefore, we suggest linagliptin monotherapy as a potential treatment strategy for HD patients with diabetes, as inflammation and atherosclerosis are important prognostic factors for these patients.

AUTHOR CONTRIBUTIONS

Kengo Kimura, Yuya Nakamura, Hitomi Hasegawa, and Masahiro Inagaki devised the study concept and design; Yuya Nakamura, Hitomi Hasegawa, Hiromichi Gotoh, and Yoshikazu Goto collected the data; Yuya Nakamura analyzed the data; Yuya Nakamura interpreted the data; Kengo Kimura, Yuya Nakamura, Hitomi Hasegawa, and Masahiro Inagaki searched the literature; Kengo Kimura, Yuya Nakamura, and Hitomi Hasegawa drafted the manuscript; Hitomi Hasegawa, Mayumi Tsuji, Tatsunori Oguchi, Hiromichi Tsuchiya, and

Masahiro Inagaki provided substantial revisions to the manuscript; and Katsuji Oguchi gave final approval of the manuscript.

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AUTHOR DISCLOSURES: None of the authors have any conflicts of interest to disclose.

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decline predicts cardiovascular mortality in hemodialysis patients. Ther Apher Dial. 2014;18:9–18.

Table 1 Baseline patient characteristics

Parameter	Mean ± standard error
Age	65.5 ± 2.8
Sex (male)	16
Diabetes treatment period (years)	10.4 ± 2.1
Dialysis treatment period (years)	6.0 ± 1.9
Body mass index (kg/m^2)	21.9 ± 0.8
Smoking (n)	6
Antiplatelet drugs (n)	9
Vasodilator drugs (n)	2
HMG-CoA reductase inhibitors (n)	6
Angiotensin receptor blockers (n)	3
Hemoglobin (g/dL)	10.6 ± 0.3
Albumin (g/dL)	3.5 ± 0.1
Potassium (mEq/L)	4.4 ± 0.2
Phosphorus (mg/dL)	4.9 ± 0.2
Urea nitrogen (mg/dL)	55.8 ± 3.6
Serum creatinine (mg/dL)	9.2 ± 0.5
High-sensitivity C-reactive protein (mg/dL)	0.2 ± 0.1

HMG-CoA = hydroxymethylglutaryl coenzyme A

Table 2 The response of oxidative stress markers to linagliptin therapy

Parameter	Before therapy	1 month	3 months	6 months
ADMA (nmol/mL)	0.64 ± 0.03	0.66 ± 0.03	0.69 ± 0.04	0.70 ± 0.03
8-OHdG (ng/mL)	0.34 ± 0.02	0.37 ± 0.02	0.32 ± 0.02	0.38 ± 0.03

ADMA = asymmetric dimethylarginine; 8-OHdG = high sensitivity 8-hydroxydeoxyguanosine

FIGURE LEGENDS

Figure 1. Superoxide dismutase (SOD) levels before and after starting linagliptin. The patients' SOD levels decreased significantly from 8.8 ± 0.5 U/mL at baseline to 7.0 ± 0.5 U/mL after 6 months of linagliptin therapy. Values are expressed as mean ± standard error. *P < 0.05.

Figure 2. Nitric oxide (NO) levels before and after starting linagliptin. The patients' NO levels increased significantly from 64.5 ± 6.6 μmol/L at baseline to 104 ± 15.4 μmol/L after 3 months of linagliptin therapy, and the NO levels remained significantly elevated at 6 months. Values are expressed as mean ± standard error. *P < 0.05.

Figure 3. Nitric oxide synthase (NOS) levels before and after starting linagliptin. The patients' NOS levels increased significantly at 3 months after starting linagliptin (57.2 ± 11.3 μg/mL vs. 31.6 ± 5.5 μg/mL), and the NOS levels increased further to 94.2 ± 13.2 μg/mL after 6 months of linagliptin therapy. Values are expressed as mean ± standard error. *P < 0.05.

Figure 1

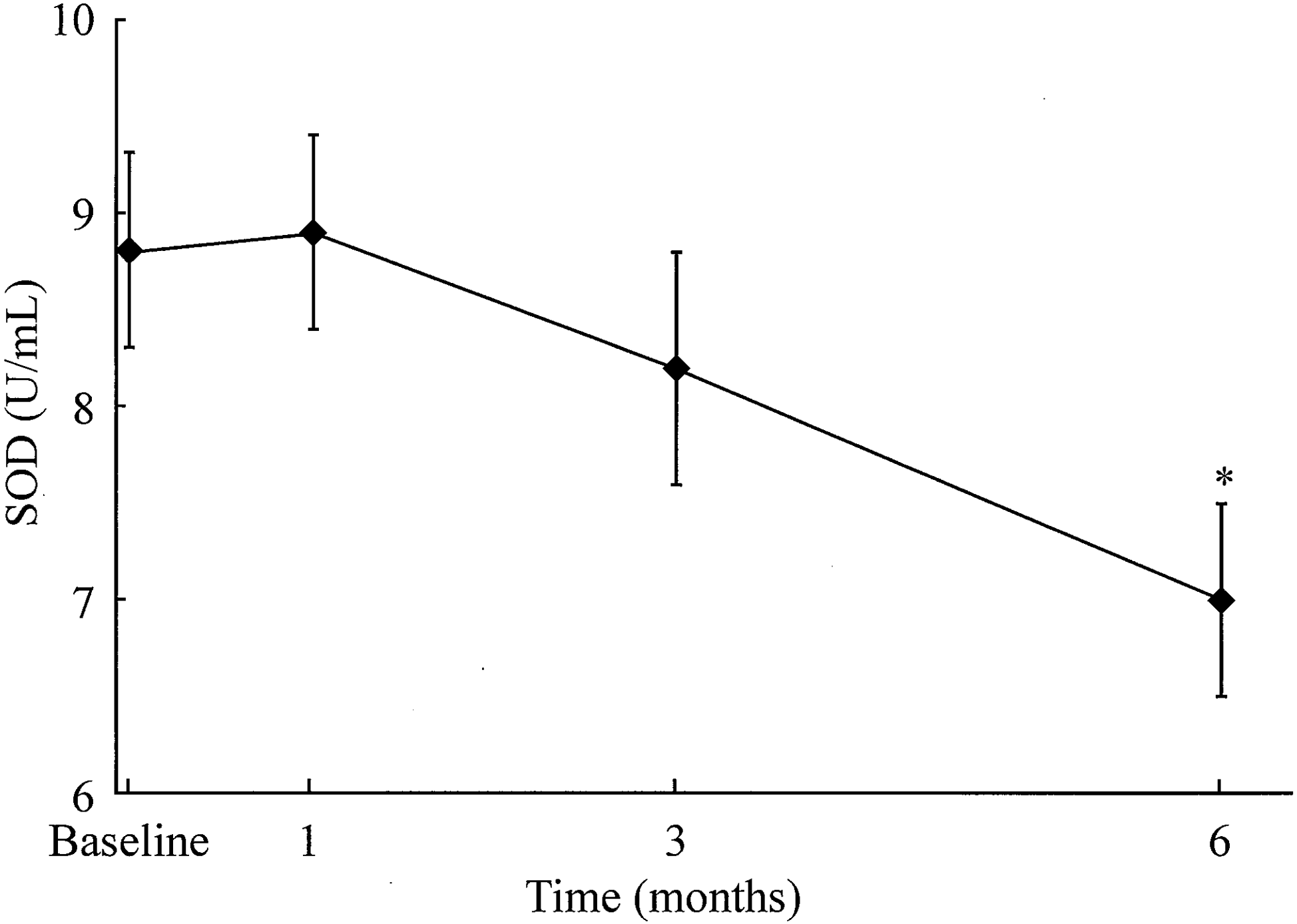


Figure 2

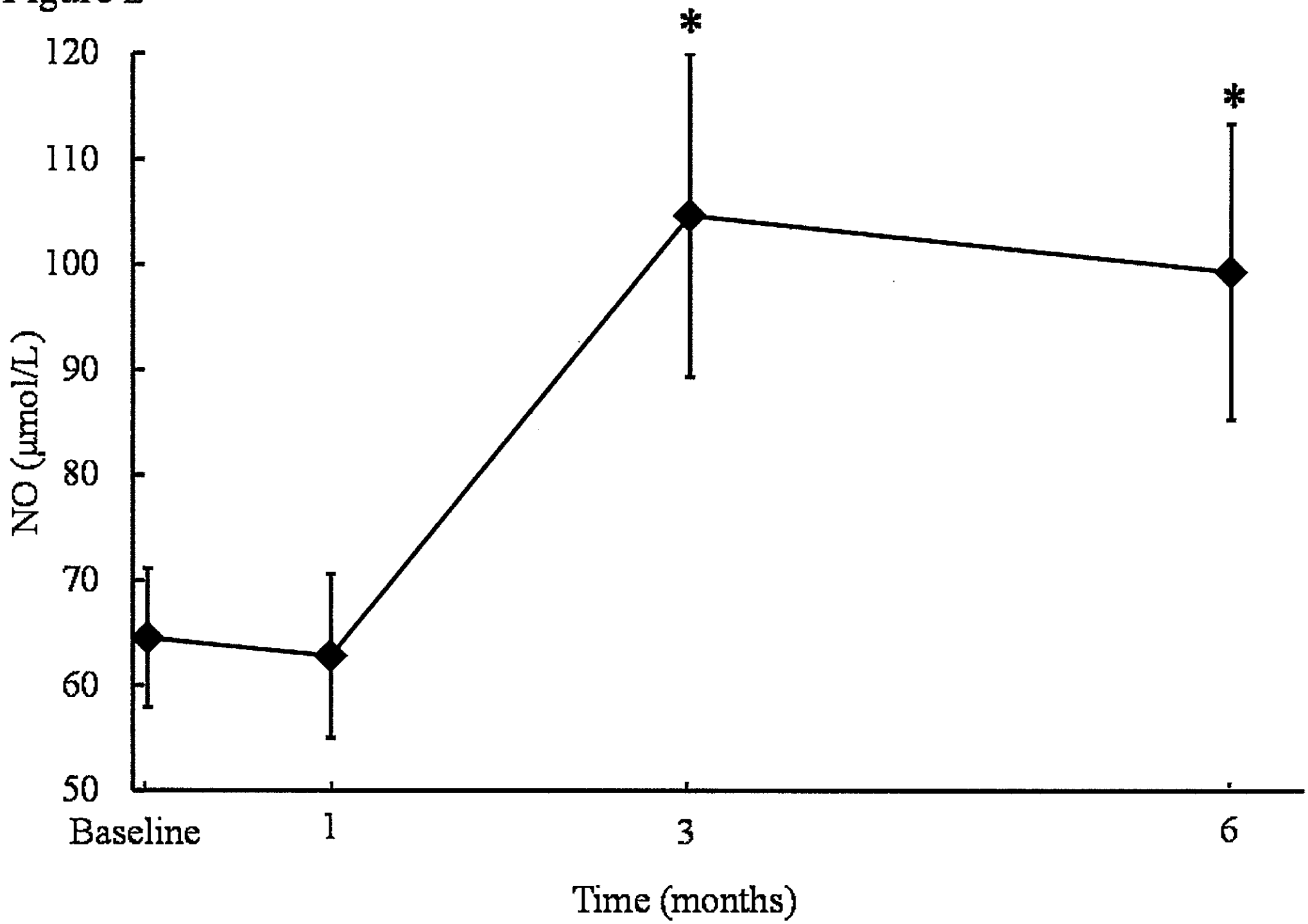
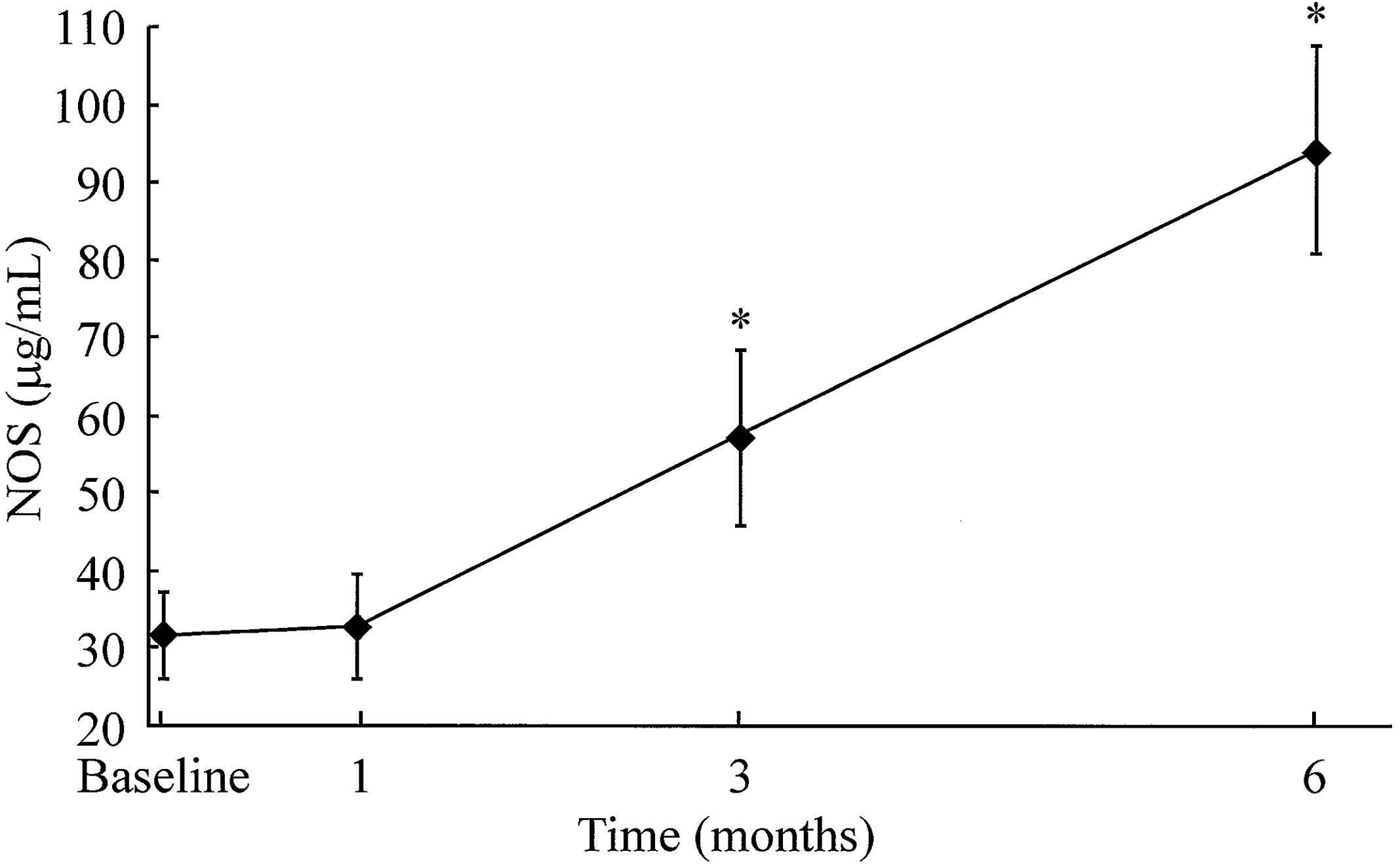


Figure 3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