DMD Fast Forward. Published on November 15, 2007 as doi:10.1124/dmd.107.018879

DMD #18879

Title page

The potential influence of CO_2, as an agent for euthanasia, on the pharmacokinetics of basic compounds in rodents.

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Running title page

a) Running title:

Influence of CO_2 on rodent PK

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c) Number of text pages: 14

Number of tables: 3

Number of figures: 6

Number of references: 26

Number of words in Abstract: 188

Introduction: 352

Discussion: 1332

d) List of nonstandard abbreviations:

AUC, area under the drug concentration time curve; CD, cervical dislocation; CL, plasma clearance; CP, cardiac puncture; MeCN, acetonitrile; IS, internal standard; V_{ss}, steady state volume of distribution

Abstract

Rodent tissue distribution and pharmacokinetic studies were performed on basic compounds Org A and Org B in support of CNS drug discovery programs. A consistent observation from these studies was that drug concentrations in plasma obtained by cardiac puncture after CO_2 euthanasia were markedly higher compared to other sampling methods (serial sampling, isofluorane anaesthesia or cervical dislocation). Further investigations demonstrated that CO_2 euthanasia led to a reduction in blood pH in both rats and mice which was not observed with the other sampling methods. The use of CO_2 euthanasia resulted in a decrease in the brain:plasma ratio of Org B, largely as a result of increased plasma concentrations. The pharmacokinetics of a basic drug, raloxifene, in rat were also influenced by sampling technique. CO_2 euthanasia prior to sampling, resulted in a 2-3 fold increase in AUC, decrease in CL and decrease in steady state volume of distribution compared to isofluorane anaesthesia. It is proposed that a decreases in the pH of blood relative to that of other tissues, as a consequence of CO_2 exposure, results in a redistribution of basic compounds out of the tissues, leading to higher concentrations in plasma.

Introduction

For tissue distribution studies or combined tissue distribution/pharmacokinetic studies in rodents, a common approach is to administer test compound to several groups of animals. Subsequently, at each designated timepoint, blood and tissue samples are taken from a group of animals following euthanasia. A survey of the literature indicates that various methods of euthanasia are used for these types of studies including CO_2 inhalation (Sauer et al., 1997; Doran et al., 2005; Becker and Liu, 2006; Venkatakrishnan et al., 2007), pentobarbital (Groll et al., 2005; Washington et al., 2000), isofluorane (Gustafson et al., 2006), halothane (Hepburn et al., 2001) and decapitation (Hong et al., 2006). In many cases the method of euthanasia was not reported.

At Organon, various CNS discovery programs have entailed the study of the pharmacokinetics and brain distribution of drug candidates in both mice and rats. Until recently, the typical sampling design of these studies in our laboratory had been as follows. For rat pharmacokinetic studies, serial blood samples were taken from a tail vein with a terminal sample taken by Cardiac Puncture (CP) following CO_2 euthanasia. For rat and mouse brain distribution studies and mouse pharmacokinetic studies, groups of animals were sacrificed at various timepoints (CO_2) prior to blood sampling (CP) and tissue harvesting.

Data from several unconnected studies in our laboratory suggested that, for some basic compounds, the different methods of sample collection (CP versus tail vein)

resulted in differences in the observed drug concentration in plasma. Whilst the dependence of drug concentration on sampling site is well-established (Chiou, 1989) this could not adequately explain all of the observations. An alternative hypothesis was that the use of CO_2 prior to blood sampling resulted in alteration of physiological pH giving rise to changes in compound distribution and hence changes in plasma concentrations. Further studies were performed with the aim of investigating this hypothesis. Data was generated on 2 proprietary small molecules (Org A and Org B) as well as raloxifene (Evista^{TM}); all three compounds are basic with pKa 9.48, 9.69 and 7.33 respectively. The initial observations and the results of these follow-up studies will be described and discussed.

Materials and Methods

Materials. Raloxifene was obtained from Sigma-Aldrich (Poole, UK). Org A and Org B were synthesised at Organon Laboratories Ltd. (Newhouse, UK). Solvents and other reagents were obtained from common sources and were of reagent grade or better. For oral administration. Org A (1mg/ml) was suspended with 0.5% (v/v) gelatin in 5% (v/v) mannitol. Org B (1 mg/ml) was dissolved in isotonic saline for both oral and intravenous administration. Raloxifene (1 mg/ml) was suspended in 5% (v/v) mulgofen in isotonic saline for oral administration and dissolved in 10% (v/v) dimethyl acetamide in water for intravenous administration.

Animals. Male Wistar BRL rats (200-300 g) and male mice (ICR or MF1, 20-30 g; Harlan, Bicester, UK) were maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled room with access to water and food *ad libitum*. All procedures involving animals were governed by a Project licence granted by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

Plasma pharmacokinetics. Animals were administered with test compound either orally or intravenously and blood samples collected at predetermined timepoints. Dose levels were as indicated in the appropriate tables and figures. For rat studies, blood samples were taken from either a lateral tail vein or from an indwelling jugular vein or carotid artery cannula. For mouse studies, blood samples were taken by CP.

Multiple sampling site studies. Rats were administered orally with test compound (Org A, 10 mg/kg; Org B, 5 mg/kg). At 4 h post-dose, blood samples were obtained from a lateral tail vein and an indwelling carotid artery cannula. Following euthanasia by overexposure to CO_2, further blood samples were collected from each of the heart (CP), jugular vein, trunk and thoracic artery.

Termination method studies. Three groups of mice (ICR, n=8) were administered orally with Org A (10 mg/kg). At 1 h post-dose blood (CP) and brain samples were collected from each group following euthanasia by either cervical dislocation (CD) or CO_2 inhalation or following anaesthesia by isofluorane.

Blood gas analysis. Rats or mice (ICR), were sacrificed by one of 3 methods: (a) CD, (b) CO_2 inhalation or (c) isofluorane anaesthesia followed by CD. A blood sample was obtained by CP from each animal and assayed using a blood gas analyser (ABL500, Radiometer Ltd., Crawley, UK) to obtain blood pH and pCO_2.

Sample preparation and LC/MS/MS analysis. All blood samples were centrifuged (4000 x g for 10 min at 4˚C) to obtain plasma. Brains were collected into ice-cold phosphate buffered saline (pH 7.4), rinsed with a further aliquot of the same buffer, blotted dry on filter paper (Whatman, Maidstone, UK) and weighed. Plasma and brain samples were stored at -20˚C prior to analysis by HPLC-MS/MS. Samples were quantified using either a PE-Sciex API-365 (turbo ionspray source, 300˚C) or an API-

3000 (turbo ionspray source, 300˚C, PerkinElmerSciex Instruments, Boston, MA) quadruple mass spectrometer. Plasma samples (50 µl) were prepared for analysis by protein precipitation using 150 µl acetonitrile (MeCN) containing 100 ng/ml of an appropriate internal standard (IS). The samples were vortex-mixed, centrifuged (4000 x g for 10 min at 4˚C) and an aliquot of supernatant removed for analysis. Prior to analysis, 100 µl water was added to each supernatant sample. Brain samples were homogenised after addition of 3 volumes of phosphate buffered saline. An aliquot (200 µl) of the homogenate was removed and protein was precipitated by addition of 600 µl MeCN containing IS. The samples were vortex-mixed and centrifuged (4000 x g for 10 min at 4˚C) and an aliquot of supernatant removed for analysis. Prior to analysis, 250 µl water was added to each supernatant sample and each sample was analysed in triplicate. Samples were injected (10-50µl; CTC Analytics, Zwingen, Switzerland) onto a Phenomenex 50 x 2 mm, 5 µm Develosil C30 (Phenomenex, Macclesfield, UK) column at room temperature. The run time varied for each analyte (3.5 – 6 min). Raloxifene and Org A were eluted with a linear gradient consisting of 0.1% formic acid in water [“A”] and 0.1% formic acid in MeCN [“B”] produced by two binary pumps [raloxifene, Shimadzu LC-10ADVP (Shimadzu, Milton Keynes, UK); Org A, Perkin Elmer Series 200 micropumps (PerkinElmerSciex Instruments, Boston, MA, USA)]. Org B was eluted with a linear gradient consisting of ammonium formate in water (10 mM) [“A”] and MeOH [“B”] produced by two Shimadzu LC-10ADVP binary pumps (Shimadzu). For Org A, the initial condition was 100% “A” held for 1 min then ramped to 100% “B” over 3 min and held for 0.5 min. The initial condition was returned to over 0.5 min and then held for a further 1

min. Org B conditions started at 90% “A” held for 1 min then ramped to 100% “B” over 0.5 min and held for 0.5 min then returned to initial conditions over 0.5 min and held for a further 1 min. Raloxifene initial conditions were 100% “A” held for 1 min then ramped to 100% “B” over 1 min and held for 1 min. Initial conditions were returned to over 0.1 min and held for 0.9 min. For all analytes, the flow rate was 1 ml/min. Standards, quality control and blank samples were prepared with either plasma or brain homogenate and were identical in composition to corresponding test samples. Accuracy of standards and quality control samples was within ±20% of the nominal value. The MS/MS transitions were 497.3/126.3 (Org A), 272.1/110.25 (Org B) and 474.16/112.25 for raloxifene.

Data analysis. Data processing was performed using Excel (Microsoft, Redmond, WA, USA) and WinNonlin v5.0.1 (Pharsight, Mountain View, CA, USA). Statistical analyses were performed using Minitab release 14 (Minitab Inc., State College, PA, USA).

Results

Plasma pharmacokinetic studies. Data from a brain penetration study and a pharmacokinetic study with Org A are shown in Figure 1. Plasma samples obtained by CP following CO_2 euthanasia gave consistently higher concentrations compared to those obtained by tail venepuncture. The estimated difference in AUC was approximately 3-fold although, due to differences in the design of the two studies, no statistical comparison was performed. Data from a pharmacology study with Org A, where the same rats were sampled by two methods, are given in Table 1. The plasma concentrations at 2 h following oral administration were between 2 and 3-fold greater in the CP samples compared to the jugular vein samples across a range of doses (3 – 30 mg/kg).

Following intravenous administration of Org B to rats, the plasma concentrations from samples taken at 24 h from the carotid artery were 2-3 fold lower than those from samples taken immediately afterwards (from the same animals) by CP after CO_2 euthanasia (Figure 2).

Following intravenous administration to male MF1 mice, plasma levels of raloxifene obtained following CO_2 euthanasia were consistently higher than those obtained following isofluorane anaesthesia (Figure 3). Estimated non-compartmental pharmacokinetic parameters (sparse analysis) with the CO_2 sampling method yielded

a higher AUC, lower CL and lower V_{ss} compared to the isofluorane technique (Table 2).

Multiple sampling site studies. Further studies with Org A and Org B in rats indicated that there was a difference in the plasma concentrations between samples obtained before and after CO_2 euthanasia from a variety of sampling sites. The trend of the mean data, shown in Figures 4 and 5 was also reflected in each individual animal (data not shown).

Termination method studies. The plasma and brain concentrations obtained 1 h following single oral administration of Org B to mice (10 mg/kg) are shown in Figure 6. Plasma concentrations from mice sacrificed by CD were similar to those obtained under isofluorane anaesthesia whereas the mice sacrificed by CO_2 euthanasia showed substantially higher concentrations. The brain concentrations of Org A appeared broadly similar between the three groups. Brain:plasma ratios derived from these data were 52.8, 45.4 and 23.5 for the CD, isofluorane and CO_2 groups respectively.

Blood gas analysis studies. In both rat and mouse, the pH of blood obtained following CO_2 euthanasia was markedly lower than that obtained by either CD or isofluorane (Table 3).

Discussion

The plasma concentration-time profile for Org A (Figure 1) shows a clear difference between samples taken by CP following CO_2 euthanasia and those taken from a tail vein. Similar observations were made in a pharmacology study (Table 1) with the CP samples giving consistently higher concentrations of Org A than the jugular vein samples from the same animals. Sample site-dependent differences in drug levels have been discussed by Chiou (1989). For drugs which distribute into tissues, marked differences in concentration between arterial and venous samples have been observed. In the early (distributional) phase following drug administration, venous concentrations can be much lower due to the net uptake of drug during its passage across the tissues. During the elimination phase, with decreasing arterial concentrations, drug will redistribute out of the tissues resulting in higher venous concentrations. Since the concentrations of Org A in the CP samples (Figure 1) are consistently higher then the venous samples throughout the study, it is not clear that the phenomena discussed by Chiou (1989) could be responsible for the observations, although a more complete profile may have aided interpretation. Further, from the imprecise nature of CP sampling, it is not possible to say whether this represents arterial blood, venous blood or a mixture. Differences in plasma concentration between sampling methods were also observed during a study of the pharmacokinetics of Org B in rats (Figure 2). Concentrations of Org B were appreciably higher in a CP sample (CO_2 euthanasia) taken from the same animals

immediately after a carotid artery sample. This observation is not inconsistent with sample site-dependent differences (Chiou, 1989).

To investigate these initial observations further, the concentrations of Org A and Org B from various sampling sites were studied after oral administration to rats (Figures 4 and 5). For both compounds, concentrations from various sampling sites (both arterial and venous) taken after CO_2 euthanasia were higher than those taken prior to CO_2 euthanasia. It was thought that the carotid artery and thoracic artery samples would not be susceptible to sample site differences and that a statistical comparison would therefore be justified. The thoracic artery concentrations (post CO_2) were significantly higher than the carotid artery samples (pre-CO_2) for both Org A (4.7-fold) and Org B (3.3-fold). This suggested that other factors aside from sample site and arteriovenous differences were responsible for the observations.

Lipophilic basic drugs are characterised by high volumes of distribution as a consequence of extensive uptake into tissues. The mechanisms governing tissue uptake are thought to be an interplay between phospholipid binding and ion-trapping (Romer and Bickel, 1979; MacIntyre and Cutler, 1988; Hung et al., 2002; Siebert et al., 2004). Ion trapping occurs as a consequence of the pH differences between blood, tissues and subcellular organelles, especially lysosomes, which are maintained at a pH range of 4-5 (de Duve et al., 1974). For basic drugs, a greater proportion of the drug will exist in the protonated form in lower pH environments. Since the protonated form is considered unable (or only sparingly able) to cross biological

membranes, the net result is a sequestration of drug into compartments of lower pH. Clearly then, a change in the pH of blood, tissues or lysosomes has the potential to alter the distribution characteristics of an ionisable drug as has been reported in several laboratories (Lüllmann et al., 1985; Shibasaki, 1989; Ishizaki et al., 1998). Indeed, modulation of distribution via alterations in pH has been proposed as a means of enhancing the efficacy of anticancer drugs (reviewed by Kaufmann and Krise, 2007).

In the current study, euthanasia of rats and mice with CO_2 resulted in markedly lower blood pH (Table 3) as a consequence of an increase in blood pCO_2 (data not shown). Changes in arterial blood and tissue intracellular pH in rats, in response to CO_2 inhalation, has previously been reported by Rothe (1984). In arterial blood, the fall in pH was approximately twice that observed in liver, spleen and heart muscle whereas skeletal muscle pH appeared resistant to mild acidosis. In contrast, changes in brain pH were quantitatively similar to arterial blood (Rothe, 1984). In the current study, anaesthesia with isofluorane did not appear to affect blood pH as compared to the CD group. Although isofluorane has been reported to produce acidosis in mice (Sjöblom and Nylander, 2007), this was relatively mild (blood pH 7.15) and took 2 hours to develop. Therefore a possible explanation for the higher plasma levels of Org A and Org B after CO_2 euthanasia is that the fall in blood pH, relative to tissues, results in redistribution of compound out of the tissues and acidic organelles into the blood. In a brain distribution study with Org A in mice (Figure 6), plasma levels were higher in the CO_2 euthanasia group compared to the isofluorane and CD groups. Brain tissue

levels of Org A were slightly lower in the CO_2 group although this was not statistically significant. As a consequence mainly of the increased plasma concentration, the brain:plasma ratio of Org A derived from the CO_2 group (23.5) was around 2-fold lower compared to the isofluorane (45.4) and cervical dislocation (52.8) groups. It is possible that a relatively small redistribution from multiple tissues could lead to a marked increase in plasma concentrations. Whilst brain:plasma ratios give a useful indication of the distributional properties of a drug, CSF drug levels are the preferred means, in our laboratory, of assessing CNS exposure with respect to pharmacological effect (Martin, 2004) and extrapolation to man. It would therefore be of interest to study the influence of CO_2 on drug levels in CSF in relation to plasma.

A study of the pharmacokinetics of raloxifene in mice after intravenous administration provided further evidence of the influence of CO_2. The plasma levels of raloxifene obtained after CO_2 euthanasia were consistently higher than those obtained under isofluorane anaesthesia (Figure 3) and yielded a 2-fold higher AUC ($p<0.001$). This difference was reflected in the estimated CL and V_{ss} (Table 2). Again, it is suggested that exposure to CO_2 immediately prior to sampling, and the concomitant fall in blood pH, resulted in a redistribution of this basic compound out of the tissues leading to higher observed plasma levels.

A series of observations from unconnected studies and subsequent follow-up investigations has provided evidence that different methods of blood sample

collection can lead to differences in the data obtained. The results of these investigations led us to suspect that the use of CO_2 euthanasia in tissue distribution and pharmacokinetic studies on basic compounds could yield potentially artefactual plasma concentration data. We are aware of several literature reports where CO_2 euthanasia has also been employed during tissue distribution or pharmacokinetic studies on other basic compounds (Handal et al., 2002; Debruyne et al., 2003; Polli et al., 2003; Meririnne et al., 2004; Doran et al., 2005; Liu et al., 2006; Venkatakrishnan et al., 2007). In one of these reports (Meririnne et al., 2004), rat tissue samples were obtained following decapitation under light sedation with CO_2. It was stated that preliminary tests demonstrated that tissue concentrations (including blood and brain) of 4-methylaminorex were not affected by light exposure to CO_2. Since these data were not reported it is difficult to make comparisons with data obtained in the current study (Figure 6) and assess the possible difference between light sedation and euthanasia. Nonetheless, the results presented here suggest that a re-evaluation of data generated from studies employing CO_2 anaesthesia or euthanasia may be warranted. Further studies would be required to clarify the mechanisms responsible for the observations described in this paper including investigating the effect of CO_2 on pH and drug distribution in various tissues. Studies on compounds with a wider range of basic pKa's and lipophilicities as well as acidic compounds are also indicated. Although not thought to be relevant to the compounds in the present study, the potential influence of CO_2 on reversible metabolism should be considered in future work of this type. The data presented here have prompted a change to isofluorane anaesthesia in our laboratory.

Acknowledgments

The authors would like to acknowledge the assistance of members of the Department of Pharmacology, Organon Laboratories Ltd., Newhouse in carrying out this work.

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Footnotes

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Legends for figures

Figure 1.

Plasma concentrations of Org A following single oral administration to male Wistar BRL rats (10 mg/kg). Open symbols represent samples taken by tail venepuncture (serial sampling, mean ± SD, n=4) and closed symbols represent samples taken by CP following CO_2 euthanasia (terminal sampling, mean ± SD, n=4 at each timepoint).

Figure 2.

Plasma concentrations of Org B following single intravenous administration to male Wistar BRL rats (3 mg/kg, mean ± SD, n=3). Open symbols represent samples taken from the carotid artery and the closed symbol represents a sample taken by CP following CO_2 euthanasia. *Statistically different from carotid artery sample $p<0.05$ by one-sample t-test.

Figure 3.

Plasma concentrations of raloxifene following single intravenous administration to male MF1mice (1 mg/kg, mean ± SD, n=4 per timepoint). Open symbols represent samples taken under isofluorane anaesthesia and closed symbols represent samples taken following CO_2 euthanasia.

Figure 4.

Plasma concentrations of Org A from various sampling sites at 4h after single oral administration to male Wistar BRL rats (10 mg/kg, mean ± SD, n=4). Samples were taken either pre- (shaded bars: TV tail vein; CA carotid artery) or post- (open bars: CP cardiac puncture; JV jugular vein; TR trunk; TA thoracic artery) CO_2 euthanasia. *Statistically different from CA, $p<0.05$, one-sample t-test.

Figure 5.

Plasma concentrations of Org B from various sampling sites at 4h after single oral administration to male Wistar BRL rats (5 mg/kg, mean ± SD, n=4). Samples were taken either pre- (shaded bars: TV tail vein; CA carotid artery) or post- (open bars: CP cardiac puncture; JV jugular vein; TR trunk; TA thoracic artery) CO_2 euthanasia. *Statistically different from CA, $p<0.05$, one-sample t-test.

Figure 6.

Mean (± SD) concentrations of Org B in male ICR mouse plasma (open bars) and brain (shaded bars) at 1 h after single oral administration (10 mg/kg). Samples obtained following euthanasia by either CO_2 inhalation (n=8) or CD (n=5) or under isofluorane anaesthesia (n=8). *$p<0.01$ compared to CD or isofluorane group (Analysis of variance, Tukey's method).

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Tables

Table 1. Plasma concentrations of Org A at 2 h following single oral administration to male Sprague Dawley rats (mean ± SD, n=4) at various dose levels.

Dose (mg/kg)	Plasma concentration (ng/ml)	
	CP following CO_2 euthanasia	Jugular vein cannula
3	202 ± 106**	66 ± 18
5	437 ± 151***	143 ± 53
10	866 ± 163***	437 ± 75
30	3346 ± 979***	1640 ± 335

Statistically different from jugular vein group by one-sample t-test; **p<0.01, ***p<0.001

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Table 2. Non-compartmental pharmacokinetic parameters of raloxifene estimated following single intravenous administration to male MF1 mice (1 mg/kg, n=4 per timepoint). Samples were taken by CP after either CO_2 euthanasia or isofluorane anaesthesia.

	Sampling method	
	CO_2	Isofluorane
$AUC_{0\text{-}7.5\ h}$ (ng.h/ml); (95% confidence intervals)	334*** (317, 351)	161 (147, 176)
CL (ml/min/kg)	49	98
Vss (l/kg)	2.7	9.0

*** Statistically different from Isofluorane, $p<0.001$; z-test following sparse analysis in WinNonlin

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Table 3. pH and gas composition of mouse and rat blood taken by CP following either CO_2 euthanasia, isofluorane anaesthesia or cervical dislocation (CD; mean ± SD).

	Mouse			Rat		
	CO_2	Isofluorane	CD	CO_2	Isofluorane	CD
n	8	7	2[¶]	8	8	8
pH	6.74 ± 0.03[†††]	7.23 ± 0.08	7.19	6.68 ± 0.11[***]	7.32 ± 0.04	7.39 ± 0.13

¶ due to technical difficulties
Significantly different from both isofluorane and CD groups (***, $p<0.001$) or isofluorane group ([†††], $p<0.001$); analysis of variance, Tukey's method.

Figure 1

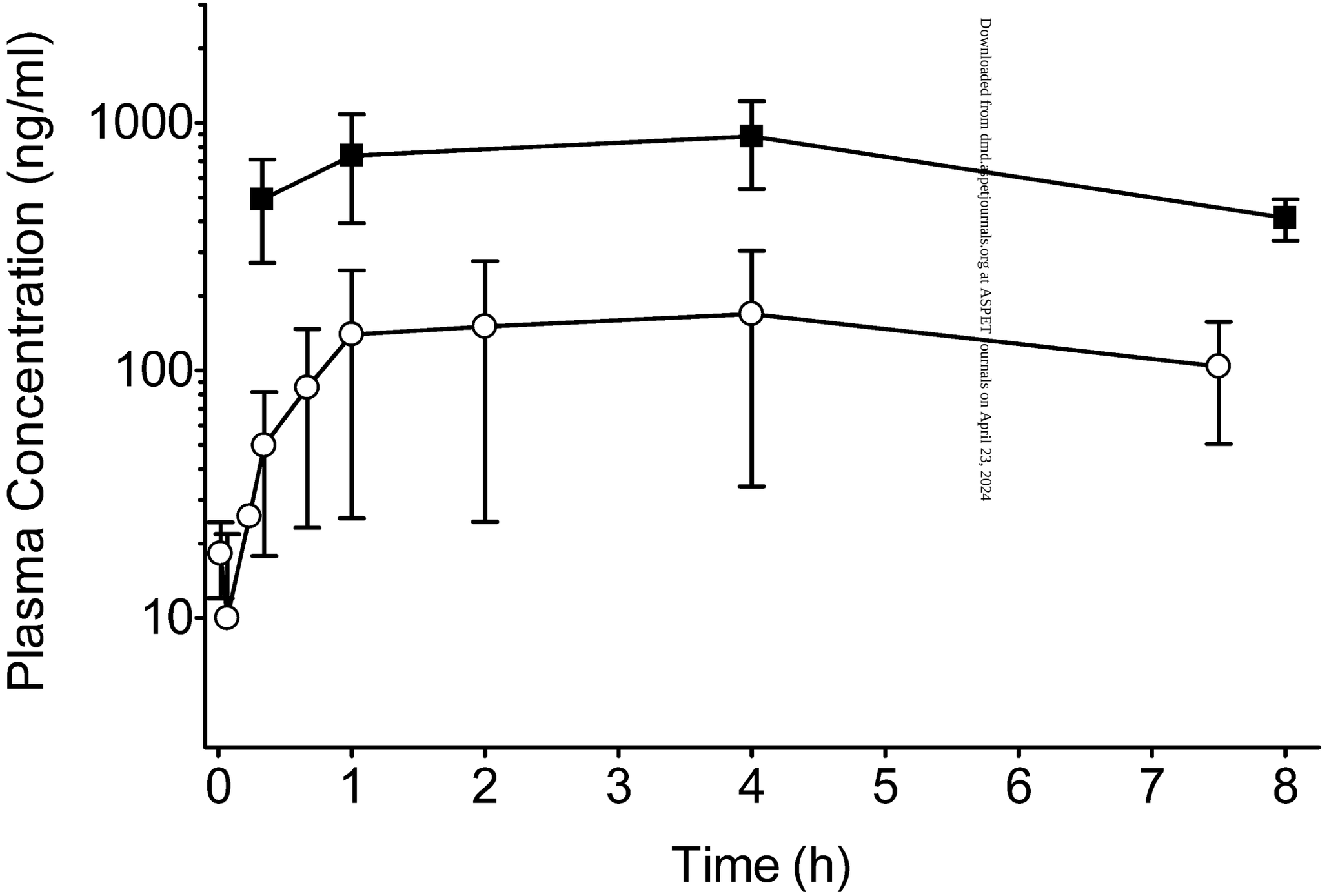

Figure 2

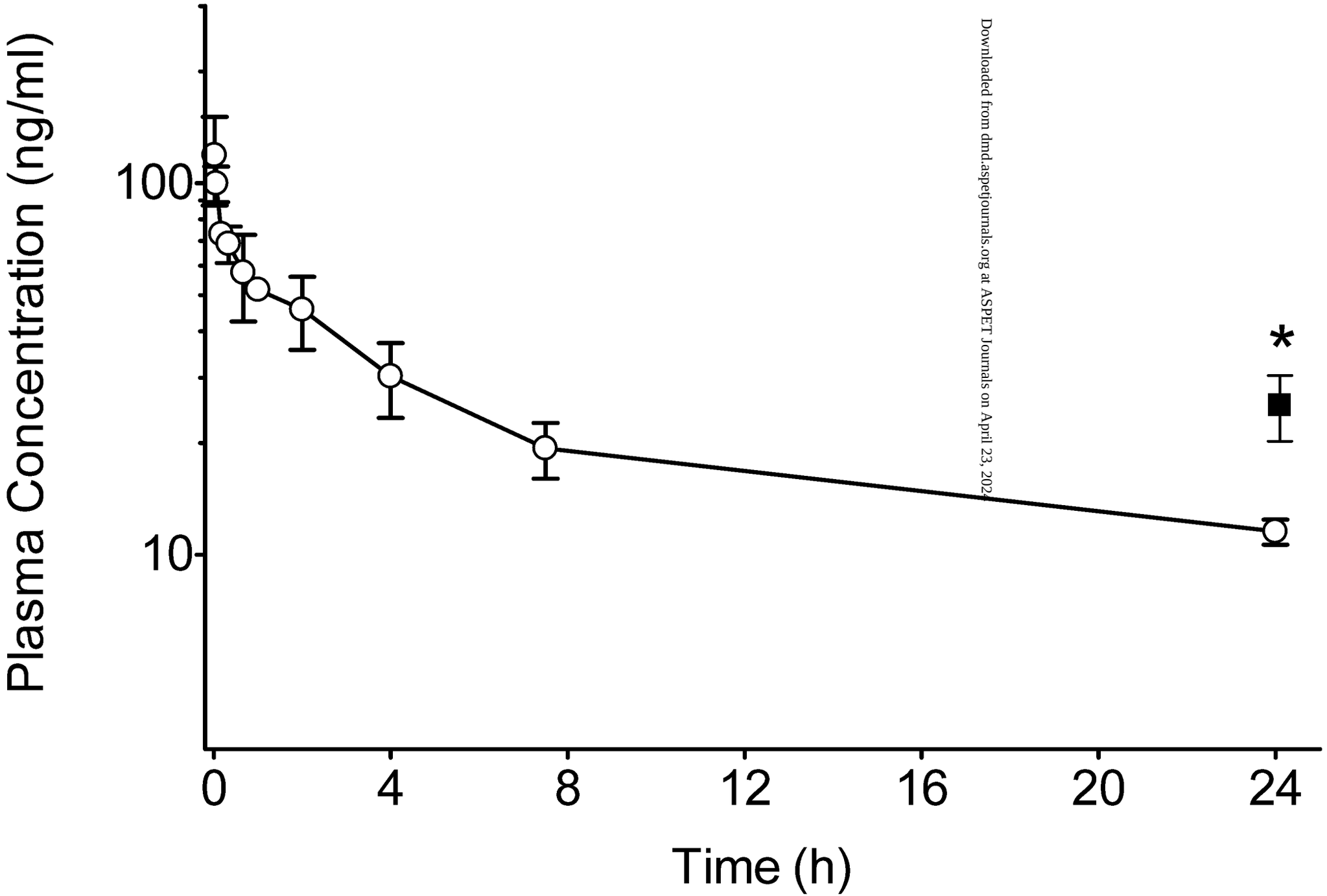

Figure 3

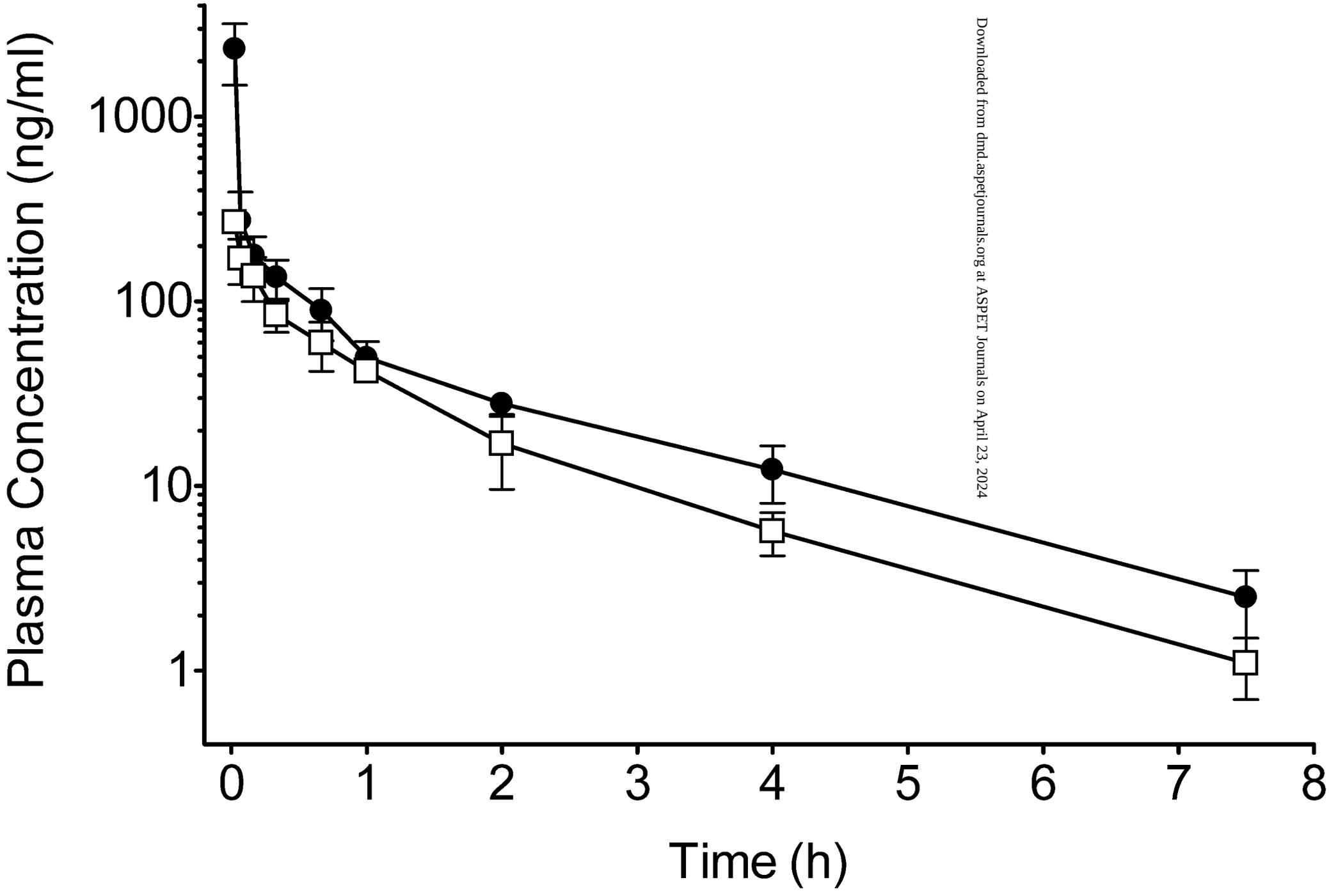

Figure 4

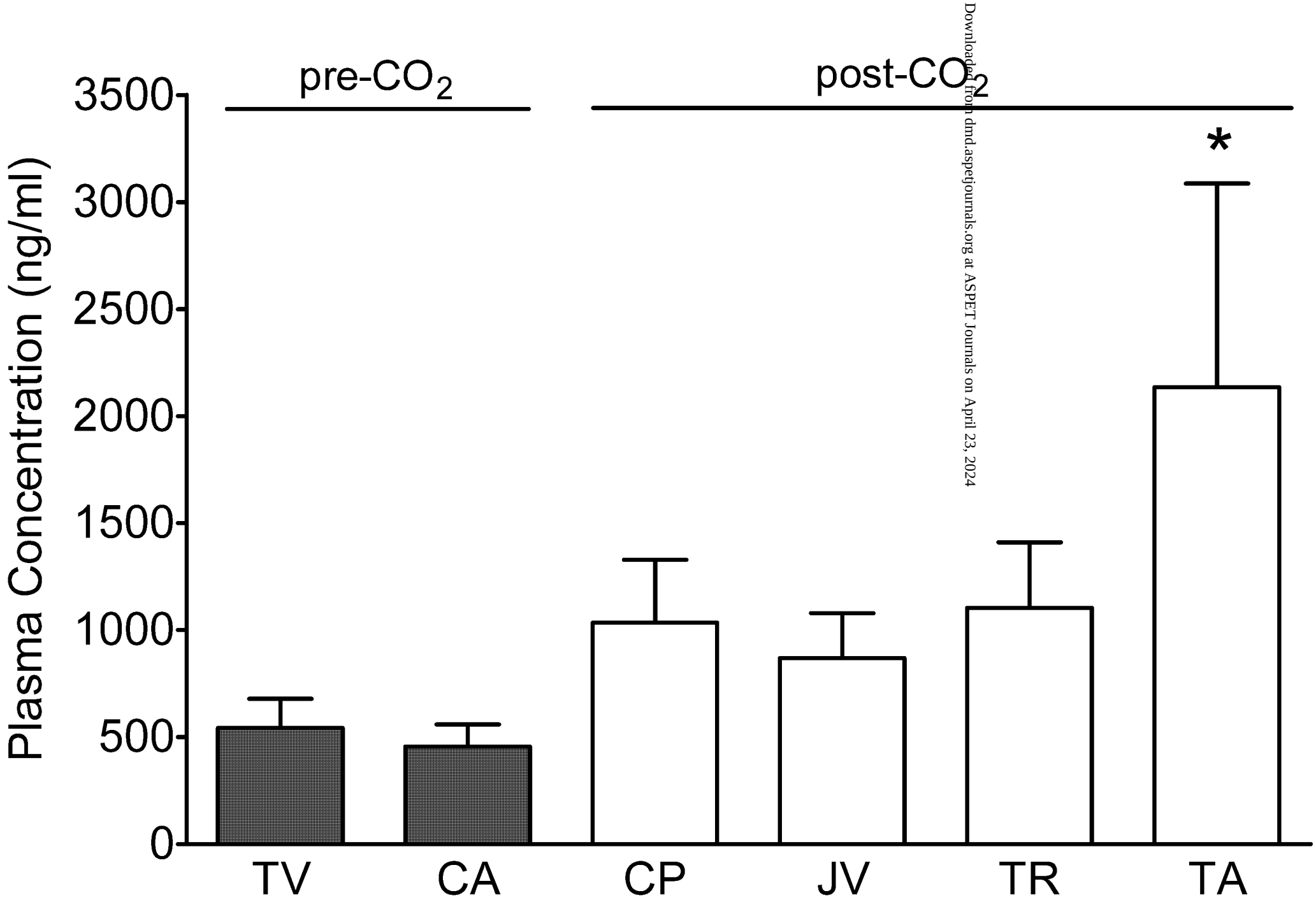

Figure 5

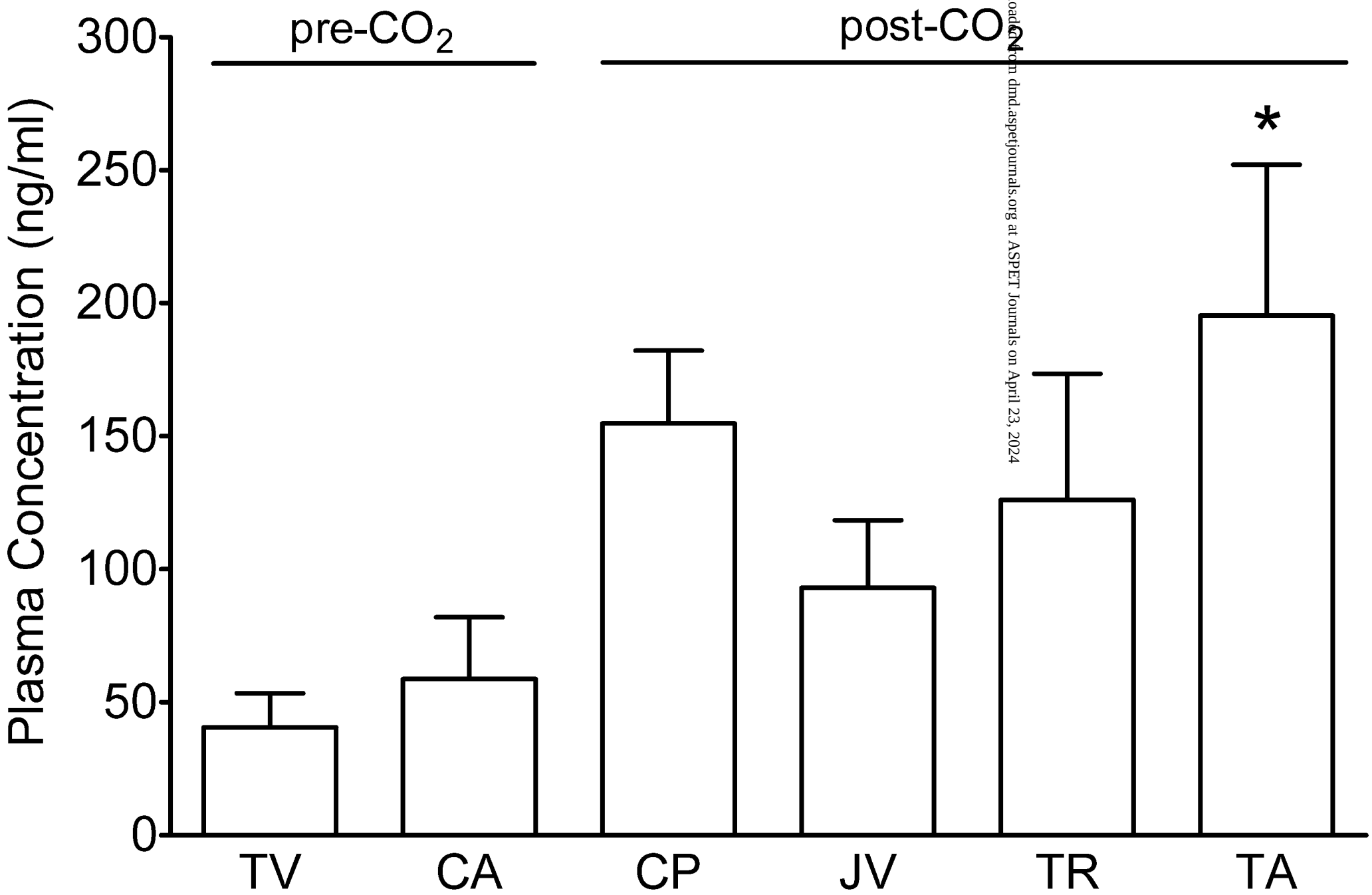

Figure 6