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Development of a novel method for monitoring the antioxidative effect of ascorbic acid in rat blood

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Abstract

A real time evaluation method for the *ex vivo* antioxidative effect in rat blood after administration of ascorbic acid (ASA) is described. The antioxidative effect in small amounts of samples (0.5 μL) could be measured by hyphenated semi-micro flow injection analysis-chemiluminescence detection and microdialysis methods. Antioxidative effects of blood microdialysates obtained from rats administered 50 mg/kg ASA through different routes (intravein: i.v., intraperitoneal: i.p. and *per os*: p.o.) were evaluated. The increased effects were maintained up to 360 min after administration. The $AUC_{0\text{-}360}$ (antioxidative effect % × min) were 16,260 ± 1,739 (i.v.), 12,873 ± 2,152 (i.p.) and 6,668 ± 2,251 (p.o.).

Additionally, ASA was determined to be the source of the microdialysate antioxidative effect by diminishing its effect with ascorbic acid oxidase. Furthermore, the concentration of ASA in the microdialysate was correlated with the antioxidative effect (r = 0.915).

Keywords: Antioxidative effect, ascorbic acid, semi-micro flow injection analysis, microdialysis, rat, chemiluminescence

1. Introduction

Recently, the benefit of consuming diets with antioxidative effects, either as food additives or as pharmaceutical supplements, against oxidative stress caused by reactive oxygen species (ROS) has become widely understood. Several studies have suggested a positive association between the consumption of antioxidative foods and their possible role in preventing chronic degenerative diseases (Engelhart et al, 2002; Klipstein-Grobusch et al., 1999). The increasing use of health foods such as dietary supplements suggests an urgent requirement to monitor the antioxidative effects for quality control purposes. Additionally, evaluation of *in vivo* antioxidative activities of health foods or their ingredients is very important to assess the disease progression, evaluate damage from oxidative stress, and provide evidence of the generation of oxidative stress in organisms. For this purpose many studies for human and experimental animals have been performed (Dolinsky & Dyck, 2011; Machado et al., 2011; Vanzin et al., 2011; Tiwari et al., 2010; Chen et al., 2009; Wilcken et al., 2000). For these reasons, simple, rapid, sensitive and selective evaluation methods of *in vivo* antioxidative effects are required.

Various parameters such as thiobarubituric acid reactive substances (TBARS) (Bakirel, Bakirel, Keles, Ulgen & Yardibi, 2008; Wang et al., 2008; Agostinho et al., 2007; Vanzin et al., 2011; Chen et al., 2009), superoxide dismutase (SOD) (Wang et al., 2008; Machado et al., 2011; Tiwari et al., 2010; Wilcken et al., 2000), catalase (Cat) (Wang et al., 2008; Machado et al., 2011; Tiwari et al., 2010; Chen et al., 2009) and glutathione peroxidase activities (Wang et al., 2008; Tiwari et al., 2010) and total radical-trapping antioxidant potential (TRAP) (Agostinho et al., 2007; Delwing et al., 2005; Shin et al., 2007; Bregano et al., 2009) have routinely been used in order to evaluate the *ex vivo* antioxidant activities of health foods and their ingredients. However, analytical methods for these parameters need time-consuming pretreatment or/and measurement during assay, and thus high-throughput monitoring of antioxidative effects is not achieved.

Microdialysis (MD) is a powerful technique in preclinical study to collect free-form substances such as medicament, nutrients, neurotransmitters or internal components in biological fluid or tissue (Osborne & Hashimoto, 2006; Hara, Mizukami, Kuriiwa &

Endo, 2009). Collection of compounds crossing the MD probe semipermeable membrane makes it possible to estimate the concentration of free-form compounds in tissues, including blood and brain. Furthermore, the clean-up procedure of the sample can often be omitted. However, since the sample size for MD is generally in the sub- or microliter range with low sample concentrations, a sensitive analytical method is required (Nakashima, Wada & Nakashima, 2005). Several studies on pharmacokinetic drug-drug interaction were previously performed using a combination of MD and high-performance liquid chromatography (HPLC)-fluorescence (FL) methods with fluorescence labeling (Tomita, Nakashima, Wada & Nakashima, 2007; Wada et al., 2008). Ueda *et al*., utilized the combination of electron spin resonance spectroscopy and an MD method, and evaluated the quenching effect on nitroxide radicals (Ueda, Doi, Nagatomo & Nakajima, 2007; Ueda et al., 2009; Ueda, Nakajima & Oikawa, 2010).

In our previous study, a flow injection analysis with chemiluminescence detection (FIA-CL) method was reported for evaluation of *in vitro* antioxidative effects and applied to health foods (Ikeda, Wada, Nishigaki & Nakashima, 2009). Using the FIA-CL method, a sensitive, rapid and precise measurement was achieved. Furthermore, a minimized FIA-CL method as a semi-micro FIA-CL (SMFIA-CL) method on peroxynitrite was established to reduce the consumption of sample and CL reagents (Wada et al., 2011). The aim of this study is to develop a real time evaluation method for the *ex vivo* antioxidative effect in rat blood after administration of ASA by hyphenating the MD and SMFIA-CL methods. Using the developed method, the antioxidative effect in blood of ASA administered through different routes (i.v., i.p. and p.o.), as well as in brain after i.p. administration, was evaluated. Furthermore, the source of the antioxidative effect was confirmed by treatment of the microdialysate with ascorbate oxidase (ASAO) and quantitation of ASA by HPLC.

2. Materials and methods

2.1. Chemicals

ASA, Cat (from bovine liver, 10,000 U/mg), SOD (from bovine erythrocyte, 90,000 U/mg), ASAO (from *Cucurbita*, 250 U/mg), 2,4-dinitrophenylhydrazine, triethylamine and acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). Luminol

from Sigma Chemical Corporation (MO, USA) was used. Peroxidase (POD, from horseradish, 285 U/mg) was obtained from Toyobo (Tokyo, Japan). Water was deionized and distilled by an Aquarius GSR-500 automatic water distillation apparatus (Advantec, Tokyo, Japan).

2.2. SMFIA system and conditions

A flow diagram of the SMFIA system for measurement of the antioxidative effect of the microdialysate is shown in Fig. 1. The SMFIA system consisted of two chromatographic pumps (LC-20 AD_{vp}, Shimadzu, Kyoto, Japan), a 7250 sample injector (Rheodyne, MA, USA), an 825-CL chemiluminescence detector (Jasco, Tokyo, Japan) and an NP20450 recorder (Rikadenki, Tokyo, Japan). A carrier solution of 60 U/L POD in 50 mM phosphate buffer (pH 7.4) was used at a flow rate of 0.12 mL/min. A solution of 425 μM luminol in 50 mM carbonate buffer (pH 9.9) at a flow rate of 0.12 mL/min was used as the CL reagent. The injection volume for the assay was 0.5 μL.

Fig. 1

2.3. MD conditions

Wistar male rats (230-310 g, Kyudo, Nagasaki, Japan) were used for experiments. Rats were anesthetized with ethyl carbamate (1.5 g/kg, i.p.) before probe implantation. A CMA MD system (Carnegie Medicine, Stockholm, Sweden) was used. The probes used for blood and brain MD were TP-20-04 cellulose membrane (4 × 0.2 mm i.d., Eicom, Kyoto, Japan) and MAB 7 polyether sulfonate (4 × 0.2 mm i.d., Eicom), respectively. The artificial cerebrospinal fluid (aCSF) consisted of 125 mM NaCl, 2.5 mM KCl, 0.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 1 mM $MgCl_2$, 12 mM $CaCl_2$, which was adjusted to pH 7.4 with 0.1 M HCl, and perfused through both probes at a flow rate of 2 μL/min. The aCSF was stored at 4°C until analysis and used after membrane filtration (Millex®-LG Sterile, 0.2 μm, Millipore Co., MA, USA). The probes were implanted within the jugular vein for blood and frontal cortex (A: +0.6 mm, L: +5.0 mm, H: +7.0 mm; according to Pakinos and Watson's brain atlas). Blood and brain microdialysates were collected before (as control) and after administration of ASA. All samples were stored at –30°C until analysis.

2.4. Calculation of antioxidative effect

The background CL generated by mixing with carrier solution and CL reagent was defined as CL_{BG}. The CL intensities obtained by aCSF, control or sample injections were defined as CL_{aCSF}, $CL_{control}$ and CL_{sample}, respectively. Thus, the percentage of quenching effect of sample on ROS in carrier and CL solutions was calculated from the following equation:

$$\text{Quenching effect \%} = \{(CL_{sample} - CL_{control})/(CL_{BG} - CL_{control})\} \times 100$$

2.5. Administration study

This experiment was performed with the approval of the Nagasaki University Animal Care and Use Committee. The dose of ASA used ranged from 10 to 100 mg/kg. Rats (n=3 for each group) were administered with a single i.v., i.p. or p.o. dose of ASA after collection of controls every 10 min for 60 min. Sampling of blood microdialysate after administration of ASA was performed every 10 min for 60 min, every 30 min for 120 min, then every 60 min for 360 min. The microdialysate was divided into two portions to measure the antioxidative effect with or without ASAO treatment. For brain MD, rats were administered a single i.p. dose of ASA after collection of controls every 5 min for 60 min. The microdialysates were collected every 5 min for 60 min, every 30 min for 120 min, then every 60 min for 300 min. The area under the curve (AUC) of the antioxidative effect for blood or brain microdialysate was manually calculated using a trapezoidal method. Data was expressed as mean ± standard deviation (SD, n = 3).

2.6. ASAO treatment

To 10 μL of blood microdialysate, 2 μL of 100 U/L ASAO in aCSF was added and then the mixture was incubated for 10 min at room temperature. The resultant solution was immediately injected into the SMFIA system to measure the antioxidative effect.

2.7. Pretreatment of microdialysate for determination of ASA

Pretreatment of the microdialysate was performed according to a previous report with minor modification (Kishida, Nishimoto & Kojo, 1992). Briefly, 5 μL of ASAO (100 U/L) in aCSF, 5 μL of 5% (v/v) phosphoric acid and 20 μL of 2% (w/v)

2,4-dinitrophenylhydrazine in 2.5 M sulfuric acid were added to 10 μL of blood microdialysate. To the resultant solution stood for 3 h at 37°C, and next 400 μL each of H_2O and ethyl acetate were added. After centrifugation (3,000 *g*) for 5 min at 4°C, 240 μL of the organic layer was removed and dried using a centrifugal evaporator (CE1, Hitachi, Tokyo, Japan). The residue was reconstituted in 200 μL of acetonitrile and applied to HPLC analysis.

2.8. HPLC system for determination of ASA

Determination of ASA in the microdialysate was performed according to a previous report (Kishida, Nishimoto & Kojo, 1992). The HPLC system consisted of an LC-10AT chromatographic pump (Shimadzu), a 7150 sample injector (Rheodyne) with a 20-μL sample loop, a Daisopak-SP-120-5-ODS-BP column (250 × 4.6 mm i.d., 5 μm, Daiso, Osaka, Japan), an SPD-10AV UV-Vis detector (Shimadzu) and an NP20450 recorder (Rikadenki, Tokyo, Japan). An acetonitrile aqueous solution (60%) containing 0.1% (v/v) triethylamine adjusted to pH 3.5 with phosphoric acid was used as the mobile phase at a flow rate of 1.0 mL/min. Absorbance of the eluent was monitored at 505 nm. A calibration curve for ASA using microdialysate spiked with known concentrations of ASA (0.25 to 5.00 μg/mL) was prepared (r = 0.994). The limits of detection (signal-to-noise ratio = 3) and quantitation (signal-to-noise ratio = 10) of ASA were 0.07 and 0.24 μg/mL, respectively. The recovery of ASA, calculated with the peak height ratio of ASA spiked to dialysate to that spiked to aCSF, was 80.1 ± 10.5% (n = 3).

3. Results and discussion

3.1. Optimization of SMFIA conditions

SMFIA conditions such as the buffer pH of the CL reagent, and the concentrations of luminol and POD, were optimized. The background CL intensity generated by mixing carrier and CL reagent (CL_{BG}), and the ratio of CL_{aCSF} to the CL_{BG} (CL_{aCSF}/CL_{BG} ratio) were examined to achieve sensitive measurement. The effects of the buffer pH (from 9.5 to 10.3) of CL reagent on CL_{BG} and CL_{aCSF}/CL_{BG} ratio were

examined (Fig. 2 (A)). Although maximum and constant CL_{BG} was given at more than 10.0, CL_{aCSF}/CL_{BG} ratio was decreased. Thus, the following experiments were done using CL solution of pH 9.9. Next, the effects of luminol concentration (275-450 μM) on CL_{BG} and CL_{aCSF}/CL_{BG} ratio were examined (Fig. 2 (B)); 425 μM luminol, which gave maximum CL_{BG} and CL_{aCSF}/CL_{BG} ratio, was used in the following experiments. Finally, POD concentration was studied in the range of 20-70 U/L. As shown in Fig. 2 (C), CL_{BG} increased with increasing POD concentration; 60 U/L of POD was selected because maximum CL_{aCSF}/CL_{BG} ratio was obtained.

In this study, a reagent for ROS generation was not used in order to simplify the SMFIA system. Antioxidative effect on the weak luminescence caused by ROS in the carrier and CL reagents in the presence of POD (=CL_{BG}) was evaluated. This is also applicable in preclinical study for sensitive evaluation of the *ex vivo* antioxidative effect in small amounts of sample (only sub-microliter) using small experimental animals. Based on the principle of the proposed method, large amounts of sample are required using intense CL_{BG}. Moreover, to confirm what kind of ROS contributes to the generation of CL_{BG}, injection of Cat or SOD was performed. Since a decrease in CL_{BG} was not observed by Cat injection, the contribution of H_2O_2 was minimal. By injection of SOD, a dose-dependent decrease in CL_{BG} was found (Fig. 3); 9,000 U/mL dose of SOD decreased 88.6% of CL_{BG}. Therefore, it can be concluded that superoxide anions contributed mainly to the generation of CL_{BG} in the SMFIA system.

Fig. 2, Fig. 3

3.2. Administration study

The proposed method was applied to evaluate the *ex vivo* antioxidative effect of ASA in blood microdialysate after single i.v., i.p. or p.o. administration to rats. The collected analytes, which pass through the microdialysis probe implanted within the jugular vein, were examined. First, ASA was administered at 10, 25, 50 and 100 mg/kg i.v. to rats. The antioxidative effects at any dose immediately increased after administration and were maintained up till 360 min. The area under the curves ($AUC_{0\text{-}360}$, antioxidative activity % × min, n = 3) calculated by time-response profiles were 7,999 ± 1,089 (10), 12,048 ± 2,406 (25), 16,260 ± 1,739 (50) and 15,266 ± 1,041 (100 mg/kg), respectively (Fig. 4). Doses greater than 50 mg/kg indicated a steady state.

Second, antioxidative effects of blood microdialysates obtained from rats administered 50 mg/kg ASA i.v., i.p., and p.o. were evaluated. Time-response profiles for blood microdialysates are shown in Fig. 5. With respect to i.p. and i.v. administration, the antioxidative effect increased rapidly and was maintained up to 360 min, whereas the effect gradually increased in the case of p.o. administration. The $AUCs_{0\text{-}360}$ values for i.v., i.p. and p.o. were 16,260 ± 1,739, 12,873 ± 2,152 and 6,668 ± 2,251 antioxidative effect % × min (n = 3), respectively. The percentage ratios of $AUC_{0\text{-}360}$ for i.p. and p.o. to i.v. corresponded to 78.4 ± 9.5% and 39.0 ± 13.4%. Since little information on the pharmacokinetics of ASA in rat has been reported, the suitability of $AUC_{0\text{-}360}$ ratio of i.p. and p.o administrations to i.v. could not be discussed. The proportion of dietary ASA absorbed for humans is 80-90% at typical intake levels of 30-180 mg/day; however, the efficiency gradually decreases at higher doses because of the saturability of the transporter, which is present in the small intestine and acts *via* a sodium-dependent active transport mechanism (EFSA, 2004). As the half-life of ASA was long enough to maintain the antioxidative activity in rat plasma (141 ± 7 h) (Kipp & Rivers, 1987), the obtained behavior of the antioxidative effect in blood dialysate is reasonable. To the best of our knowledge, this is the first report to evaluate the antioxidative effect of blood after administration of ASA through different routes (i.v., i.p. and p.o.).

Typical recorder responses of the blood microdialysate after i.p. administration of ASA are shown in Fig. 6. High-throughput measurement of the *ex vivo* antioxidative effect was successfully achieved by the MD-SMFIA method without time-consuming pretreatment. The TBARS method requires derivatization with TBA under acidic conditions (Bakirel, Bakirel, Keles, Ulgen & Yardibi, 2008; Wang et al., 2008) and the TRAP method required measuring time in the range of 6-60 min for an assay (Agostinho et al., 2007; Delwing et al., 2005; Shin et al., 2007). Furthermore, in regards to the sample size requirement, these methods are not suitable for use in conjunction with MD to monitor *in vivo* antioxidative effects, because a sample volume of > 10 μL is required.

Fig. 4, Fig. 5, Fig. 6

To evaluate the transportation of ASA into brain tissue, brain microdialysate was obtained by implanting a probe within the frontal cortex and applied to SMFIA analysis.

The antioxidative effect of the dialysate after single i.p. administration of ASA (50 mg/kg) was measured; however, an increase in the antioxidative effect compared to control was not observed, as shown in Fig. 7. In a previous report by Hara *et al*., (2010) low transportability of ASA in brain was observed. They showed that single i.p. administration of dehydroascorbic acid resulted in an dose-dependent increase of ASA level in rat striatum. To generate a neuroprotective effect of ASA in the striatum, repeated administration of a high dose of ASA (750 mg/kg, i.p. for 3.5 days) was required (Stamford et al., 1999). Therefore, both ASA dose and administration route should be taken into consideration in evaluating the antioxidative effect of ASA in brain.

The proposed method is anticipated to be applicable for the measurement of antioxidative effects in other organs in which MD probes could be implanted. Furthermore, it may be practical for evaluating *ex vivo* antioxidative effects of other antioxidants for preclinical screening. However, this method has a number of limitations: 1) this method can be applied to compounds that can be recovered by an MD probe. The material of the probe should be considered when heavy adsorption of compound to the probe membrane is observed. 2) Due to MD implantation being highly invasive, its applicability to humans is limited. Therefore, it may be concluded that at this stage the proposed method is suitable for preclinical screening of antioxidants using small experimental animals.

Fig. 7

3.3. ASAO treatment

The source of the increased antioxidative effect observed by ASA injection was evaluated. The blood microdialysate was treated with ASAO and immediately analyzed by the SMFIA system. The representative recorder responses of dialysates corrected before and after i.v. administration of ASA (50 mg/kg) are shown in Fig. 8. Increased antioxidative effect (B) was decreased to a comparable level (C) to that of pre-administration (A) by treatment with ASAO. This result suggested that the antioxidative effect would be positively correlated to plasma ASA levels. However, the potential for increases in other antioxidative factors owing to ASA administration requires discussion. In previous reports, ASA intake resulted in improved antioxidative factors such as reduced:oxidized glutathione ratio (Tomofuji et al., 2009) and lipid peroxidation (Sanbe et al., 2009) as well as blood ASA level in rats.

However, in these cases, high doses (more than 1 g/kg) of ASA were administered for several weeks. In our study, a single administration of a low dose of ASA was used, and thus the observed antioxidative activity is caused by the injected ASA.

Fig. 8

3.4. ASA determination in microdialysate

The quantitation of ASA in blood dialysate has been achieved using an HPLC method after derivatizing dehydroascorbic acid with 2,4-dinitrophenylhydrazine (Kishida, Nishimoto & Kojo, 1992). ASA was converted to dehydroascorbic acid with ASAO. First, the recovery of ASA by the blood MD probe (TP-20-04 cellulose membrane, 4 × 0.2 mm, i.d.) was evaluated. *In vivo* ASA recovery of the probe was calculated using a standard solution of ASA in aCSF at 100 μg/mL. Recovery and loss were calculated using equations according to our previous report (Wada et al., 2008):

$$\text{Recovery \%} = C_{out} / C_{in} \times 100\%$$

$$\text{Loss \%} = [(C_{in} - C_{out}) / C_{in}] \times 100\%$$

where C_{in} is ASA concentration in the perfusate (μg/mL) and C_{out} is ASA concentration in the dialysate (μg/mL). The recovery and loss factors for calculating *in vivo* recovery of the microdialysis probe were 10.0 ± 4.6% (for $\text{Recovery}_{in\ vitro}$), 20.7 ± 2.5% (for $\text{Loss}_{in\ vitro}$) and 6.3 ± 1.1% (for $\text{Loss}_{in\ vivo}$), respectively. Subsequently, the ratios for the microdialysates calculated by the following equation were 3.0 ± 1.6% (n = 4).

$$\text{Recovery}_{in\ vivo} = \text{Loss}_{in\ vivo} \times (\text{Recovery}_{in\ vitro} / \text{Loss}_{in\ vitro})$$

Quantitation of ASA in the dialysate after administration of ASA (50 mg/kg, i.p.) was performed (n = 3). The ASA level reached maximum (2.97 ± 0.39 μg/mL) after 45 min of administration and was maintained until 360 min (Fig. 9). The concentration range of ASA was from 1.22 to 2.97 μg/mL. The antioxidative effect of blood dialysate with i.p. administration seemed to correspond to the concentration of ASA. A good correlation coefficient for 12 plots of the antioxidative effect in Fig. 5 against ASA concentration in Fig. 9, where both sampling time were corresponded, was observed (r = 0.915). This fact also supported ASA as the source of the antioxidative effect in blood.

The ASA concentration quantitated by the proposed method included oxidized forms such as dehydroascorbic acid. However, the ratio of oxidized forms in total ASA in rat plasma was estimated to be about 4.5% (Kishida, Nishimoto & Kojo, 1992).

Additionally, rats can synthesize ASA and the reported endogenous plasma concentration in Sprague-Dawley rats is 10.3 μg/mL (Kishida, Nishimoto & Kojo, 1992). The ASA concentration in the dialysate was 0.32 μg/mL, which corresponded to 10.7 μg/mL of blood based on the recovery of the MD probe (=3%). Therefore, a comparable concentration to the previous report was obtained in this study.
In our previous studies, ASA indicated intense antioxidative effects on ROS using an *in vitro* evaluation method (Ikeda, Wada, Nishigaki & Nakashima, 2009; Wada et al., 2011). However, the *in vivo* antioxidative effect is subject to the compound's pharmacokinetic properties. This is one of the main reasons that *in vivo* antioxidative effects are distinct from those observed *in vitro*. Therefore, the evaluation of *in vivo* antioxidative effects using an appropriate method is needed.

Fig. 9

In conclusion, SMFIA hyphenated with an MD method was practical for evaluating the *ex vivo* antioxidative effect of the microdialysate after ASA administration. ASAO treatment and quantitation data by HPLC supported the fact that external ASA increased the blood antioxidative effect. To support these observations, a quantitative method with sufficient sensitivity is required.

Antioxidative effects in other organs implanted with a microdialysis probe, such as brain, kidney and skin, could potentially be evaluated. The proposed method may also be applicable for the evaluation of *ex vivo* antioxidative effects of other antioxidants, showing good recovery with an MD probe, for preclinical screening.

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Figure Captions

Fig. 1 Flow diagram of SMFIA system and its conditions.

Fig. 2 Effects of buffer pH of CL reagent (A), and concentrations of luminol (B) and POD (C) on CL intensity and CL_{aCSF}/CL_{BG} ratio.

Fig. 3 Recorder responses of SOD.

Fig. 4 Antioxidative effects of blood microdialysates with ASA administration (n = 3).
Dose: 10-100 mg/kg.

Fig. 5 Antioxidative effects of blood microdialysates after ASA administration to rats (n = 3).
Dose: 50 mg/kg ASA.

Fig. 6 Recorder responses of blood microdialysates after ASA administration (50 mg/kg, i.p.) to rats.

Fig. 7 Antioxidative effects of brain microdialysates after ASA administration to rats (n = 3).
Dose: 50 mg/kg ASA.

Fig. 8 Recorder responses of blood microdialysates after ASA administration (50 mg/kg, i.p.) to rats with/without ASAO treatment.

Fig. 9 Time-concentration curve of ASA after administration to rats.

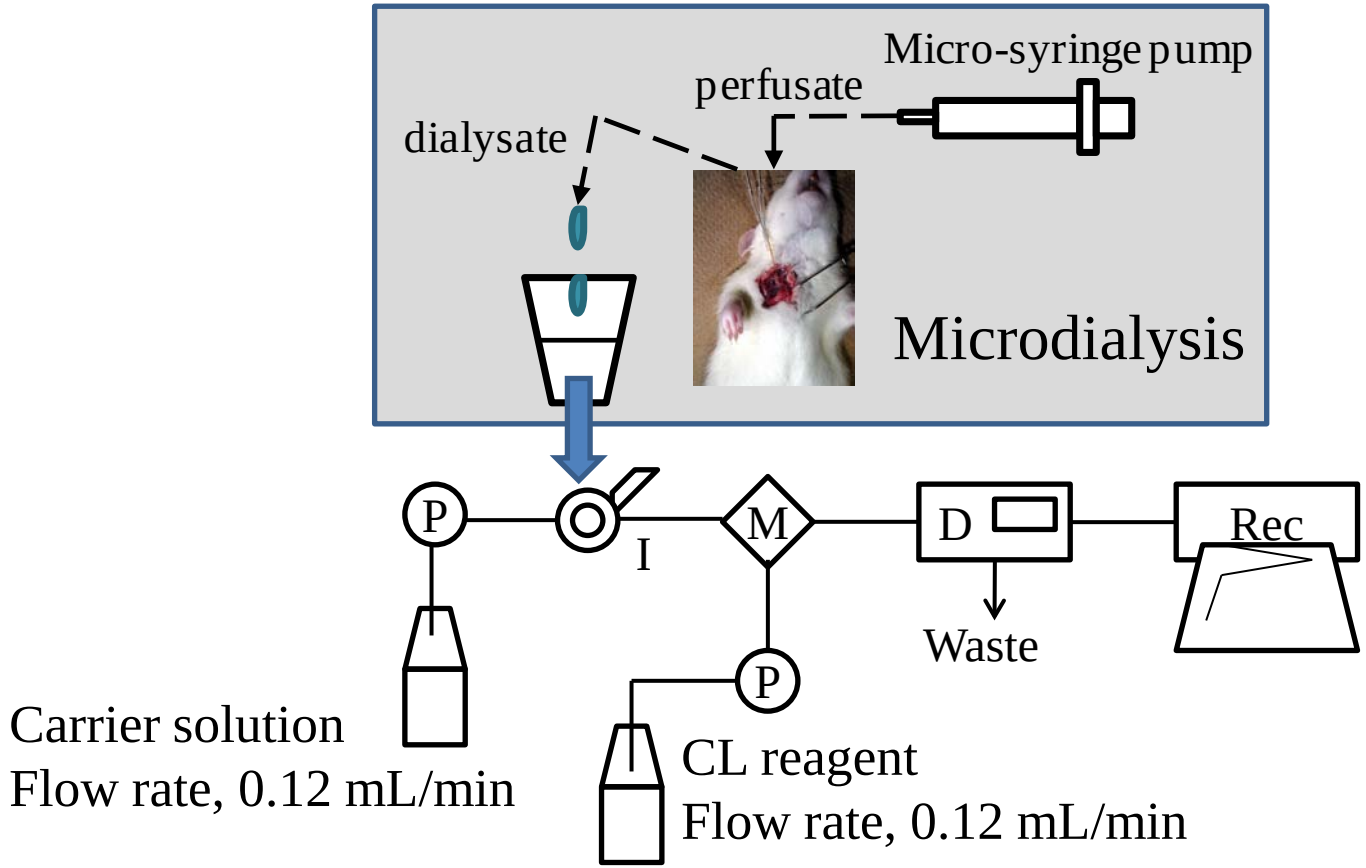
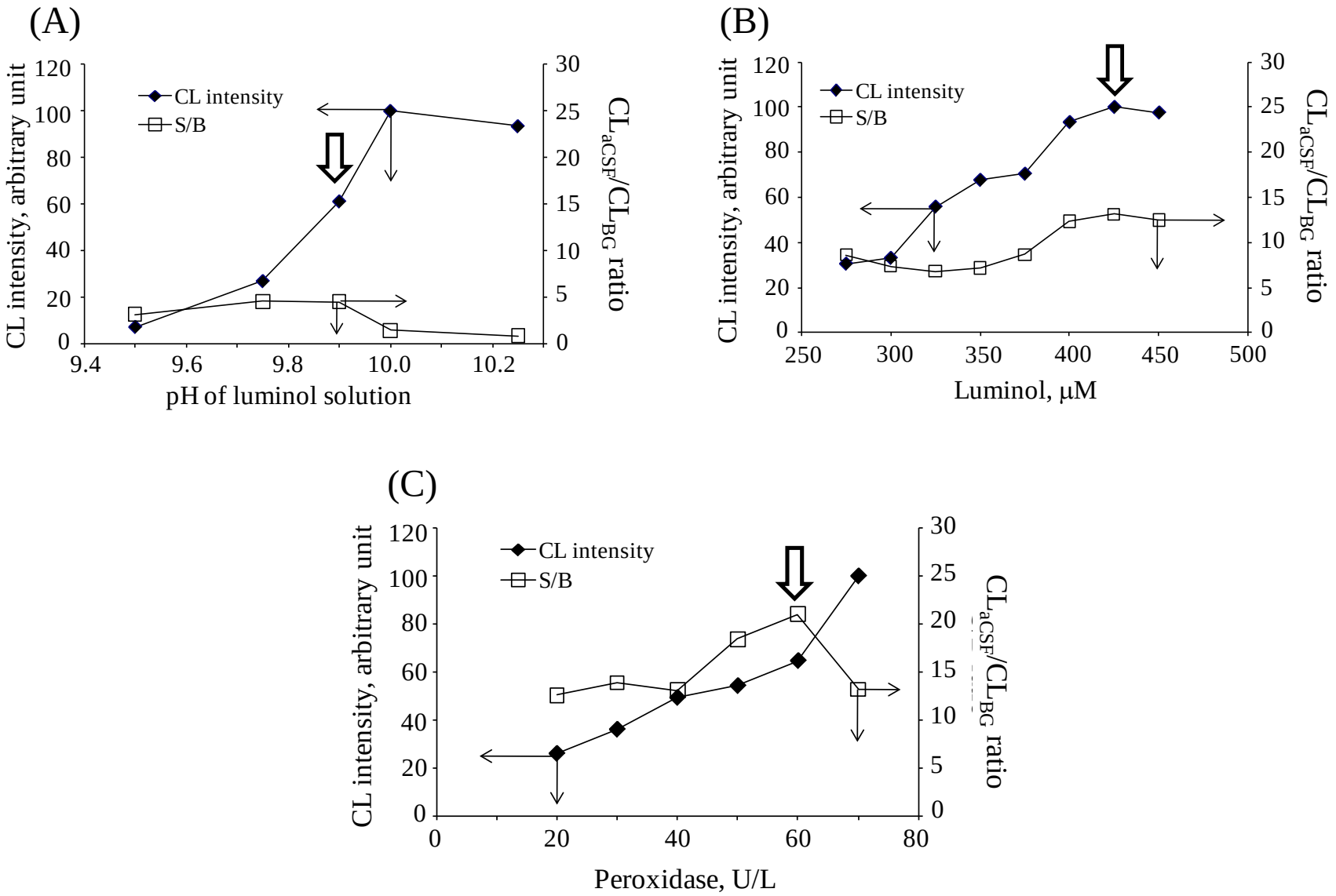


Fig. 1 K. Nakashima et al.



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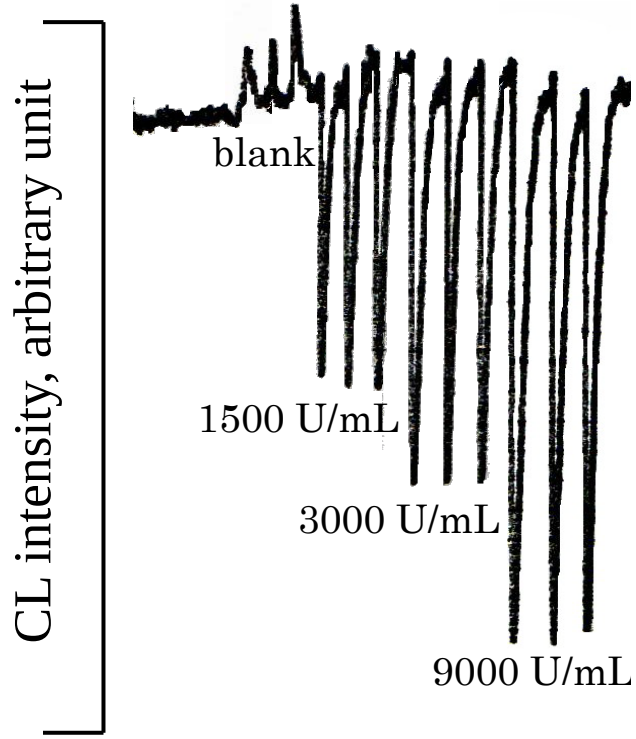

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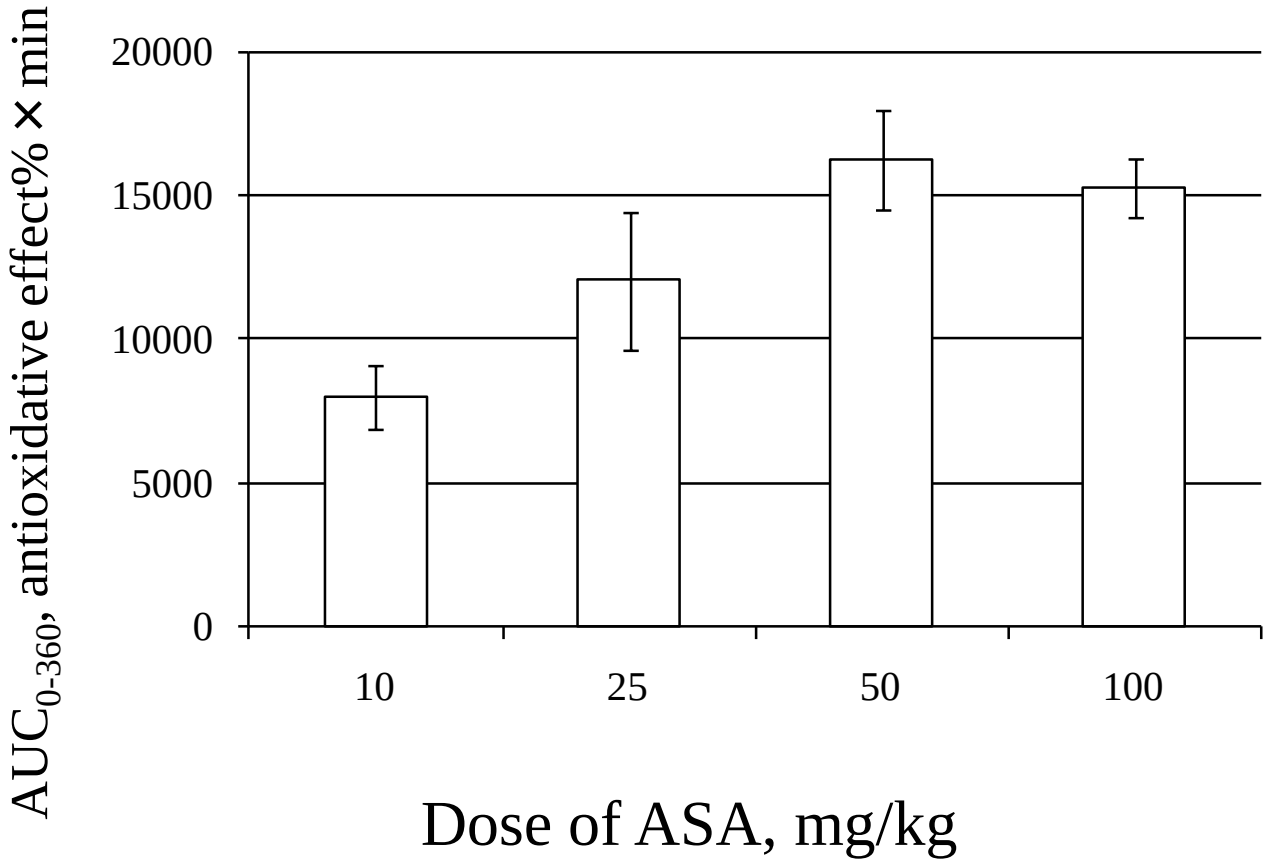

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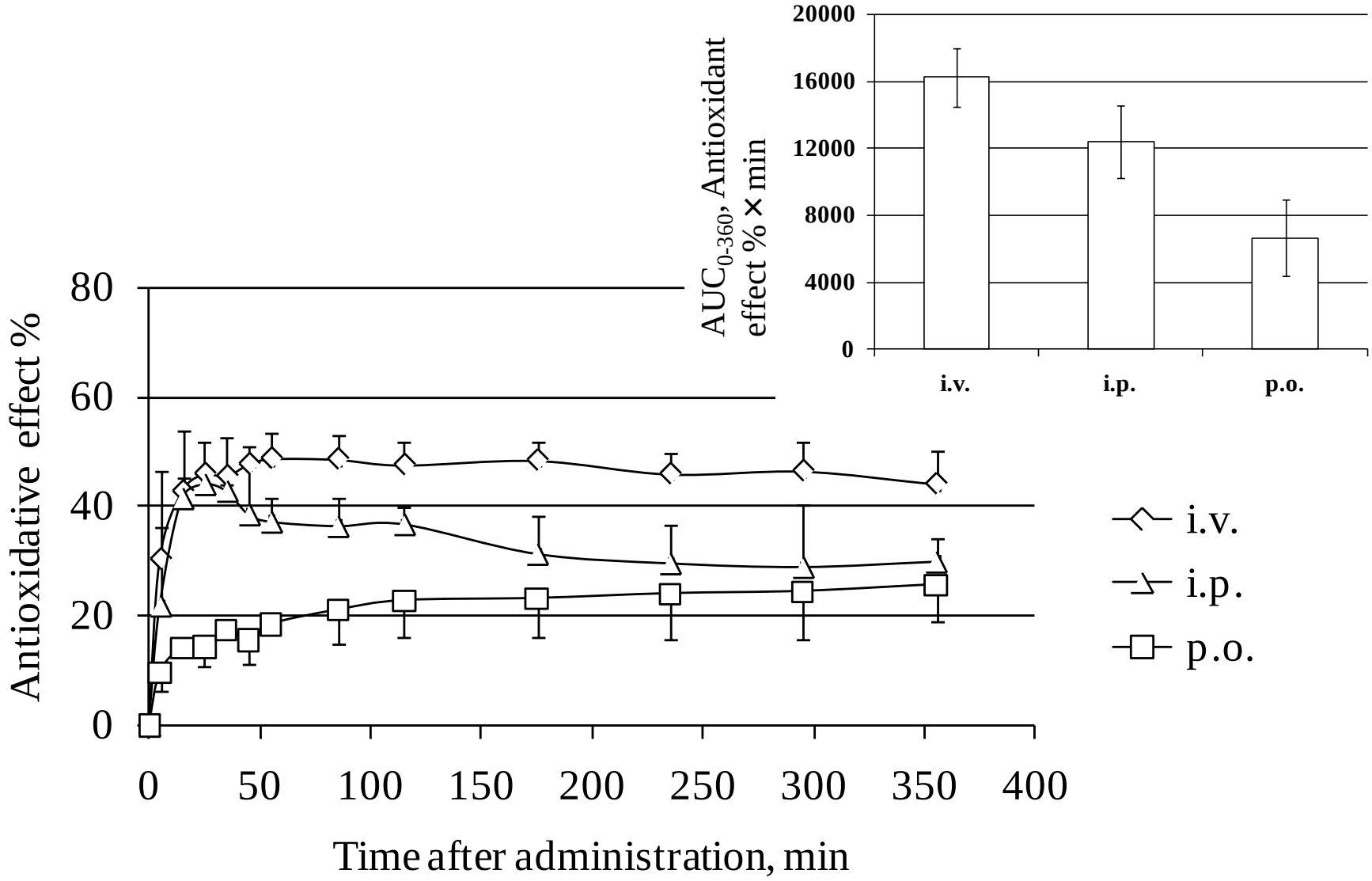

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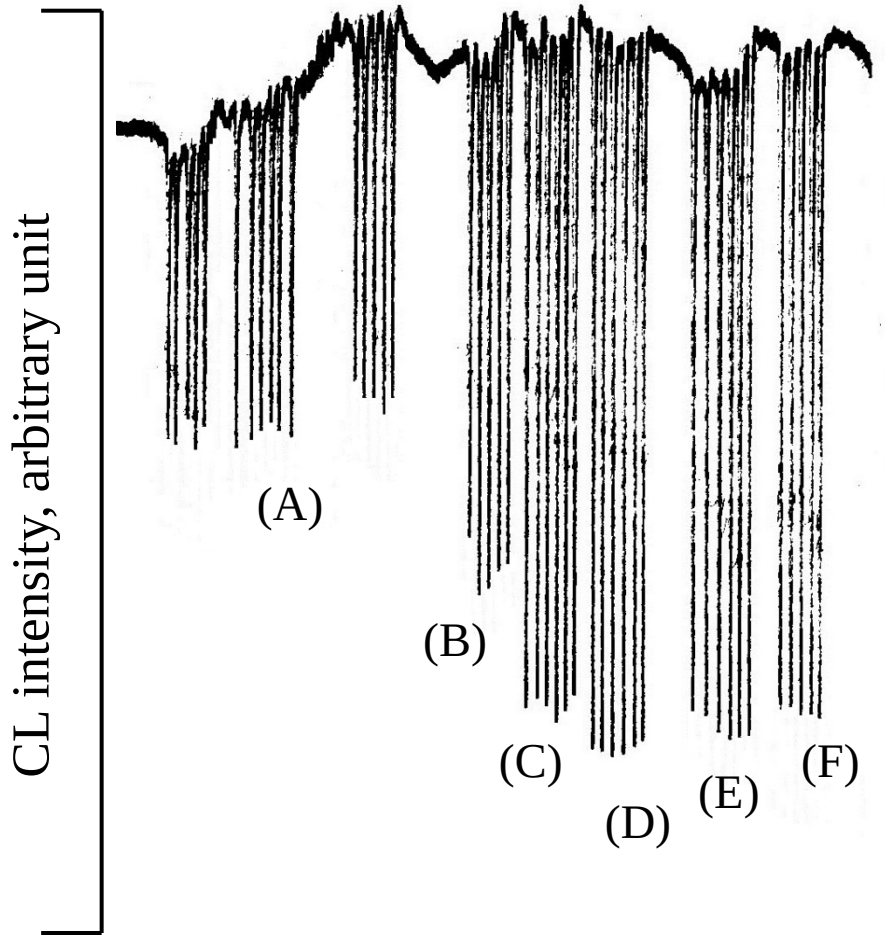

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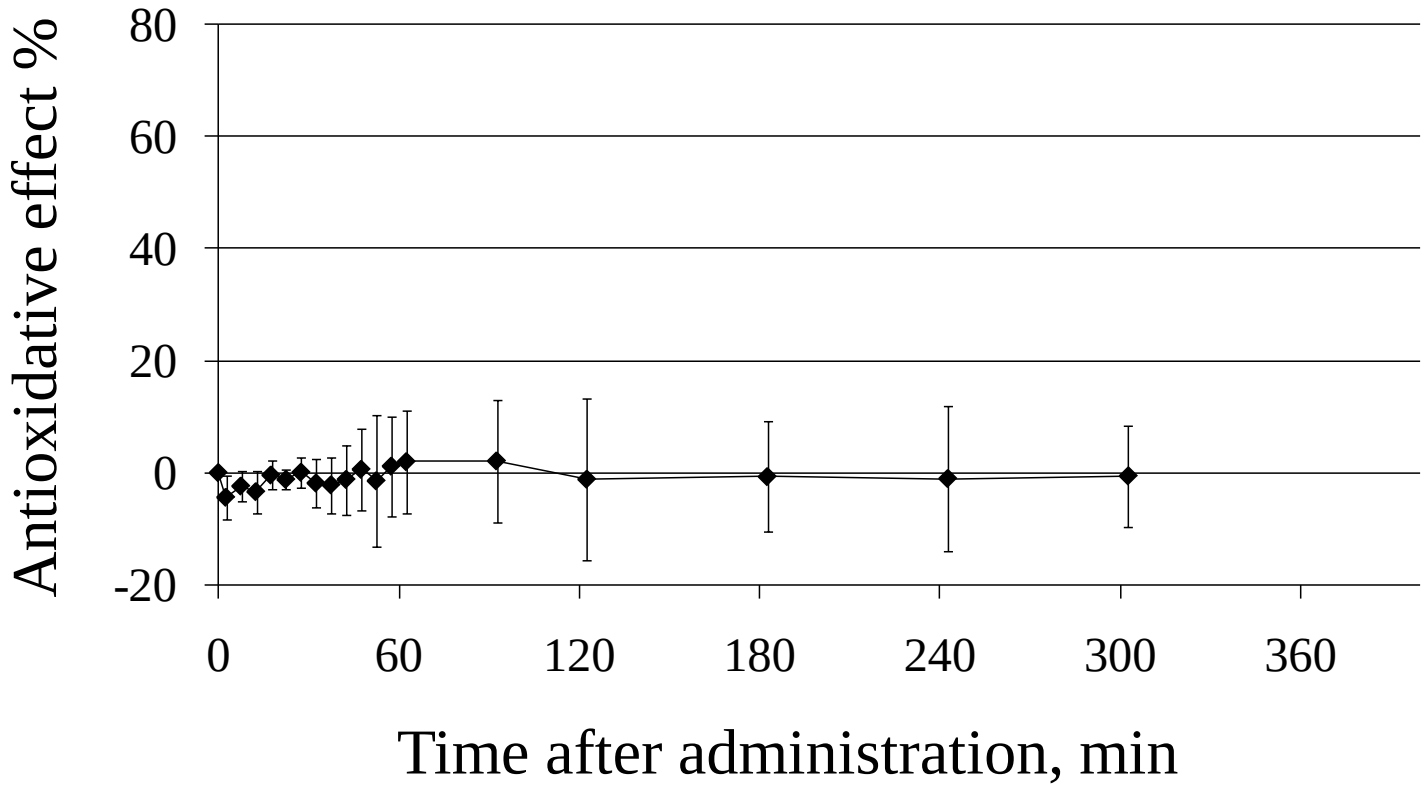

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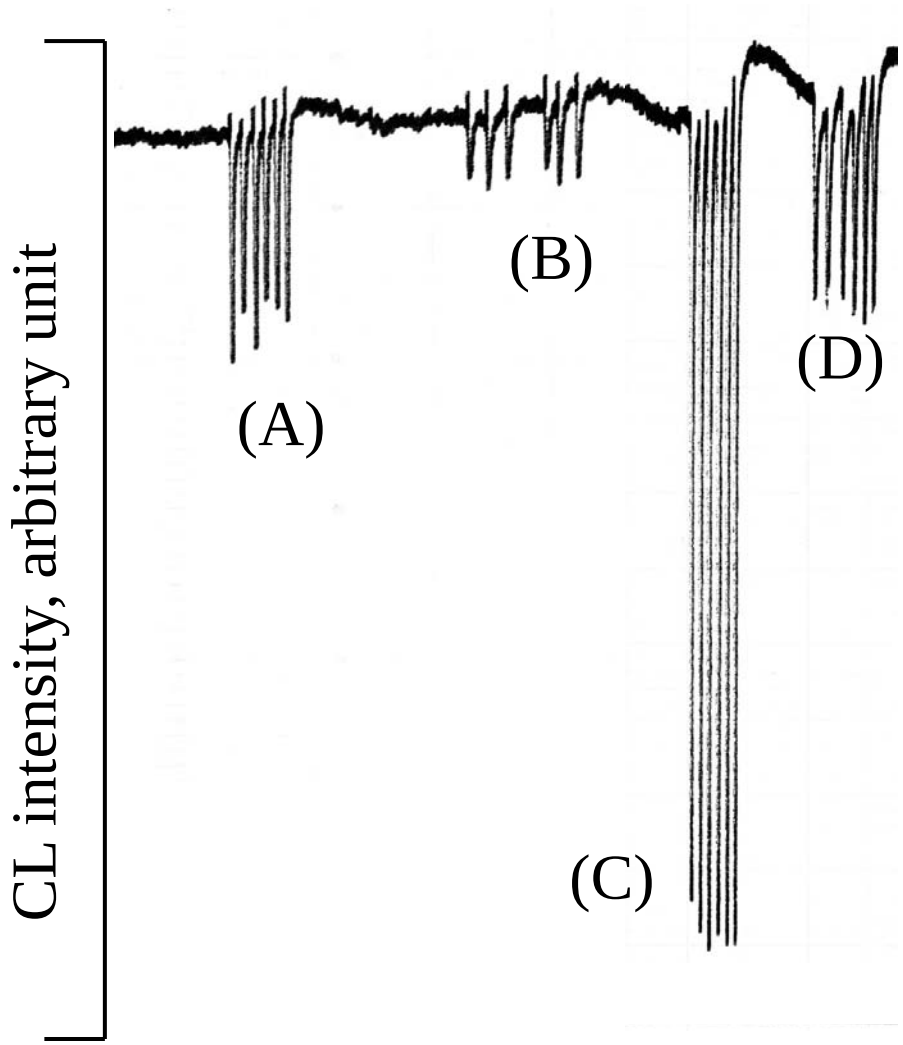

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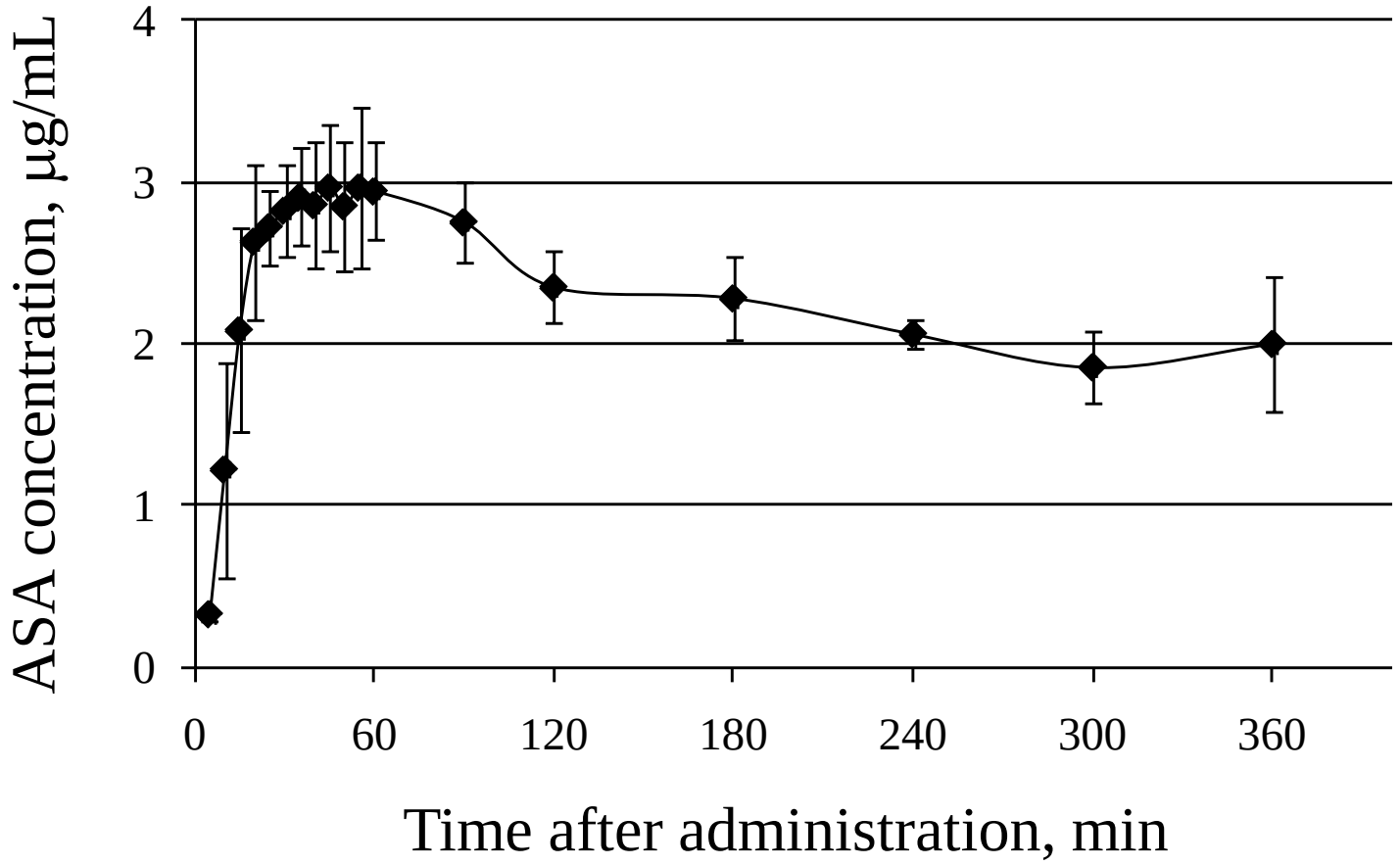

Fig. 9 K. Nakashima et al.