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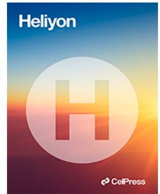

Research article

Effect of blood sample storage period on d-ROMs and BAP test data

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A R T I C L E I N F O

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A B S T R A C T

The Diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests can easily and rapidly measure the state of oxidative stress in the blood; they have been used to determine the relationship between oxidative stress and various diseases. However, the extent to which the blood storage period affects the analyzed data remains unclear. In clinical practice, the storage conditions for samples after blood collection vary. Therefore, the influence of blood storage conditions, particularly the reversible redox state, on biochemical tests has been thoroughly investigated. The storage conditions of the sample may affect its state; however, its effect on oxidative stress has not been investigated yet. In this study, considering that the time from blood collection to blood cell separation differs depending on the clinical setting, we analyzed the effect of storage period on the redox analysis data of blood samples stored for a certain period in a 4 °C refrigerator without centrifugation. Heparinized plasma samples from three healthy adult men in their 30s were subjected to the d-ROMs and BAP tests. The analysis was performed at the following 12 time points: immediately after blood collection; 1, 3, 6, 12, and 24 h later; and 2, 3, 4, 5, 6, and 7 days later. The d-ROMs and BAP values varied and were unstable after 1 h of blood collection. These findings suggest that centrifugation should be performed within 1 h after blood collection, at the latest. In a clinical setting, data should be interpreted with caution if centrifugation is performed more than 1 h after blood collection, even if heparin is added and the samples are stored at 4 °C.

1. Introduction

Reactive oxygen species (ROS), which are oxygen groups with high oxidizing power, are byproducts of essential respiratory

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metabolism and are essential in various physiological functions, such as immune responses, intracellular signaling, and pathogen defense. The ROS generated in the body are neutralized by antioxidant mechanisms involving superoxide dismutase 1 (SOD1) and glutathione (GSH). However, prolonged illness, surgery, viral and bacterial infections, and mental stress can result in excess ROS that cannot be neutralized by the antioxidant mechanisms of the body. Excessive ROS promotes the generation of harmful ROS, such as hydroxyl radicals, with strong oxidizing power. ROS oxidize biological components, such as DNA, proteins, and lipids, to result in biological dysfunction [1]. This state is called "oxidative stress," and it is greatly involved in the onset and worsening of the symptoms of various diseases. Therefore, it is important to accurately measure oxidative stress levels in the body.

The state of biological oxidative stress can be evaluated using two main methods, measuring the oxidants in the body and the antioxidant capacity [2]. The former includes assays, such as 8-OHdG (one of the oxidized DNA) measurements in urine and thiobarbituric acid-reactive substances to quantify lipid peroxide in the blood. The latter includes methods for measuring the activity of antioxidant enzymes, such as SOD1 and GSH. Although these methods provide valuable insight into oxidative stress levels, they often require relatively large blood sample sizes. In contrast, electron spin resonance (ESR) spectroscopy can be used to detect ROS, including hydroxyl radical and superoxide radicals, within living organisms. However, ESR requires specialized equipment and techniques, making it less practical for routine use. Overall, a simple, rapid, and cost-effective method for measuring the oxidative stress in living organisms is of great importance. Furthermore, particularly in clinical settings, there is a need to use minimal sample volumes to minimize invasiveness.

Among the several methods, the Diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests are simple, rapid, and breakthrough methods for evaluating oxidative stress by measuring both oxidant and antioxidant capacities using small plasma samples. d-ROMs measure the blood levels of hydroperoxide and oxidative metabolites produced by ROS [3]. BAP evaluates the reducing power of blood toward oxidants [4]. Moreover, d-ROMs and BAP tests have demonstrated correlations between oxidative stress and various diseases, including metabolic syndrome [5], smoking [6] and lung cancer [7].

The d-ROMs and BAP tests use centrifuged plasma or serum from blood samples; however, if the plasma is frozen, the test results remain stable even months after collection [8–10]. Furthermore, if blood is centrifuged immediately and the plasma removed, stable results can be obtained at room temperature for one or more days [11]. However, in clinical practice, it may not be possible to quickly separate plasma or serum and perform blood sample measurements due to other tasks (such as IV setup, vital sign monitoring, and patient care) immediately following blood collection. The relationship between specimen storage conditions and test results has been investigated using major biochemical tests on blood samples. However, this has not been considered when performing the d-ROMs and BAP tests, and these storage conditions have not been detailed in the Methods section of the respective papers, making it difficult to compare measurements across different studies.

Accurate oxidative stress assessment is crucial for diagnosing and monitoring various conditions. Since immediate plasma separation is often impractical in clinical practice, understanding how storage duration impacts test results is vital for ensuring reliable data. This study provides important insights into optimal blood sample handling, enhancing the clinical utility of d-ROMs and BAP tests.

2. Material and methods

2.1. Study design & subjects

This study was approved by the Ethics Review Committee of the Osaka University Hospital (Approval No. 22053 [T1]-3). The analysis was performed using blood samples obtained from three healthy adult men. Table 1 shows the background and analysis data. The blood samples were collected from participants with an empty stomach before noon. Heparin was added to 60 units/mL of blood, and the samples were stored at 4 °C and protected from light until measurement (Fig. 1a). The heparin concentration was consistent with the heparin content in the blood gas-measuring syringes used in clinical practice. To purify the plasma components, the blood samples were centrifuged (4 °C, 9 gravitational accelerations, 10 min). Samples for measurement were collected at the following storage periods: immediately after blood collection; 1, 3, 6, 12, and 24 h later; and 2, 3, 4, 5, 6, and 7 days later.

Table 1
Background of samples No. 1, 2, and 3. All three participants were males of the same age.

	No.1	No.2	No.3
Age(years)	35	34	35
Gender	male	male	male
Height(cm)	173	179	167
Weight(kg)	64	61	74
BMI(kg/m^2)	21.4	19.0	26.5
Abdominal circumference(cm)	76	73	82
Smoking history	+	+	+
Conditions for blood collection	after a night shift	as usual	as usual
d-ROMs(U CARR)	340	207	273
BAP(μ M)	2167	1707	1874
BAP/d-ROMs	6.37	8.25	6.86

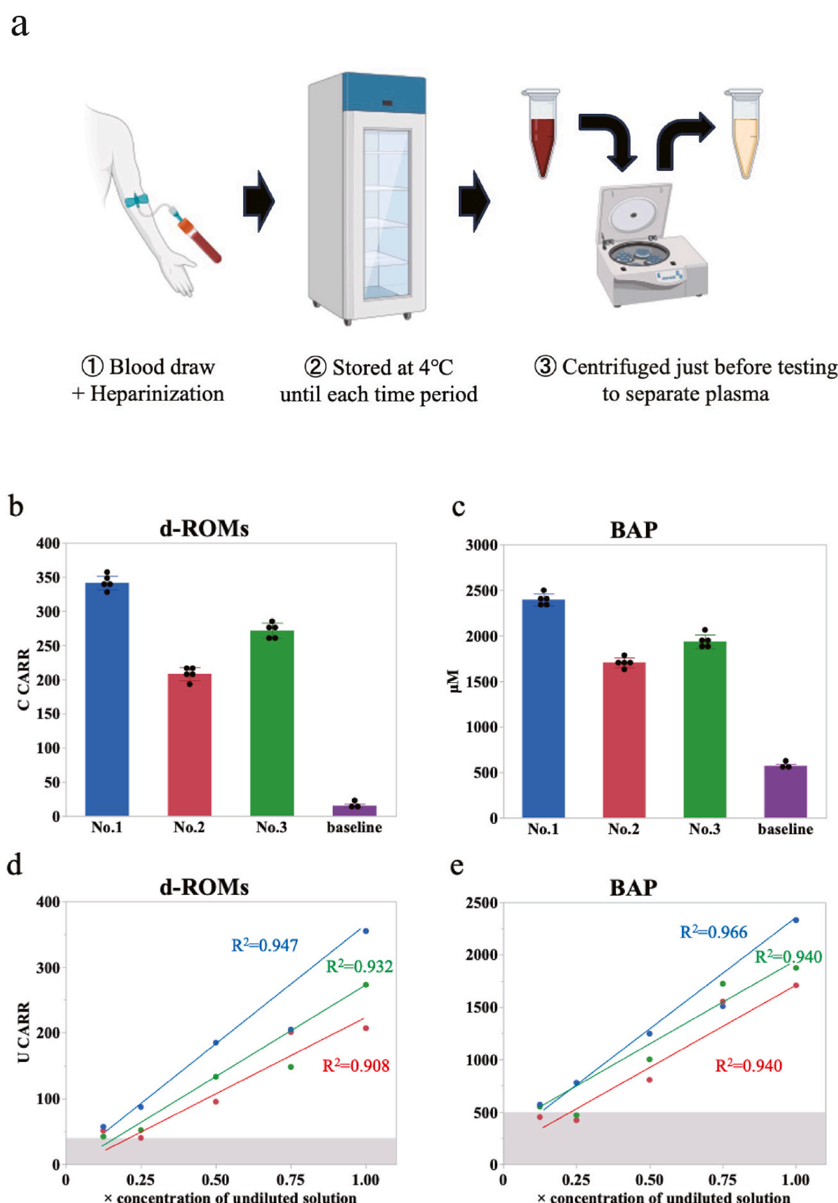

Fig. 1. Reproducibility and linearity of d-ROMs and BAP tests (a) Experimental protocol. The blood samples were stored in the dark at 4 °C until just before the test. Created using BioRender Com. (b, c) Each sample and saline as baseline control. The bar graphs represent the mean and standard deviation. (d, e) Measurements of sample diluted with saline. Blue: No. 1, red: No. 2, and green: No. 3. The grey area indicated values below the detection limits of the d-ROMs (40 U CARR) and the BAP test (500 μM).

2.2. Oxidative stress measurement and blood gas analysis

The oxidative stress level in plasma was analyzed via the d-ROMs and BAP tests using REDOXLIBLA (Wismerll Co., Ltd., Tokyo, Japan). d-ROMs and BAP indicate the amount of lipid peroxide in and the antioxidant capacity of the blood, respectively. d-ROMs values are expressed in Carratelli units (U CARR: 1 U CARR corresponds to 0.8 mg/L of hydrogen peroxide). BAP values indicate the reducing power of blood using the amount of trivalent iron ions (μM) reduced to divalent iron ions as an index. Anticoagulants commonly used during blood collection include heparin, EDTA, and citric acid, but only heparin can be used in this analysis. EDTA and citric acid cannot be used in this test because they chelate the iron ions required for both d-ROMs and BAP test. A comparative analysis was performed using d-ROMs, BAP, and the ratio of BAP to d-ROMs (index of oxidative stress). The manufacturer's recommended quantitative ranges are d-ROMs (40–1000 U CARR) and BAP (500–6000 μM) [12,13]. Blood potassium (K) and glucose levels were measured using ABL800 FLEX (RADIOMETER, Tokyo, Japan).

3. Results

3.1. D-ROMs and BAP tests are highly reproducible and linear for different concentrations

First, we performed statistical validation of d-ROMs and BAP tests. Five consecutive measurements of Sample No. 1, 2 and 3 showed high reproducibility (Fig. 1b and c). When saline was used as a baseline control, d-ROMs and BAP measurements were significantly lower than any plasma sample measurements (Fig. 1b and c). Linearity of the d-ROMs and BAP tests was demonstrated using plasma sample diluted with saline at three different concentrations (Fig. 1d and e).

3.2. D-ROMs values in blood samples remained stable for 1-h post-collection, with varying stability thereafter, particularly peaking for no. 1 and no. 2 on days 4–5

No significant differences were observed in the background information, including age, height, and weight (Table 1). However, only patient No. 3 had high BMI and abdominal circumference values, and patient No. 1 had a history of smoking.

When comparing the measurement values immediately after blood collection, all sample d-ROMs were stable for 1 h, and only sample No. 1 was stable even after 24 h (Fig. 2a). Samples No. 2 and 3 increased in size gradually, and no significant fluctuations were observed from 3 to 24 h. After the first day, the d-ROMs of all the samples fluctuated. In particular, samples No. 1 and No. 2 showed a similar pattern, reaching a peak on Day 4 and 5 and settling down to the same value as that on the 1st day on Day 6 (Fig. 3a). Even in terms of the rate of change based on the measured value immediately after blood collection, a stable d-ROMs value was observed only for up to 1 h, which was similar to the actual measured value. (Figs. 2d and 3d).

3.3. BAP levels and their rate of change remained stable for up to 24 h across samples, but started to increase from day 2; whereas BAP/d-ROMs initially stabilized for 1 h, they subsequently decreased, with significant increases observed in all samples except no. 2 from day 1

The actual measured values and rate of change in BAP did not fluctuate dramatically up to 24 h in any sample but showed an increasing trend over time from Day 2. (Fig. 2b, e, 3b, 3e). For BAP/d-ROMs, the value was stable for 1 h because of the influence of d-ROMs; however, it showed a decreasing trend after 1 h. After 1 day, the values of all samples, except No. 2, increased significantly (Fig. 2c, f, 3c, 3f).

3.4. Serum potassium increased and glucose decreased with storage time across samples, accompanied by advancing gross hemolysis

We analyzed the temporal changes in serum potassium and glucose levels in each sample, which are known to change over storage time. Potassium levels increased, whereas blood glucose levels decreased in proportion to the storage duration (Fig. 4a and b). There was little individual variation in this change, consistent with the report in parentheses. In addition, gross hemolysis advanced over time

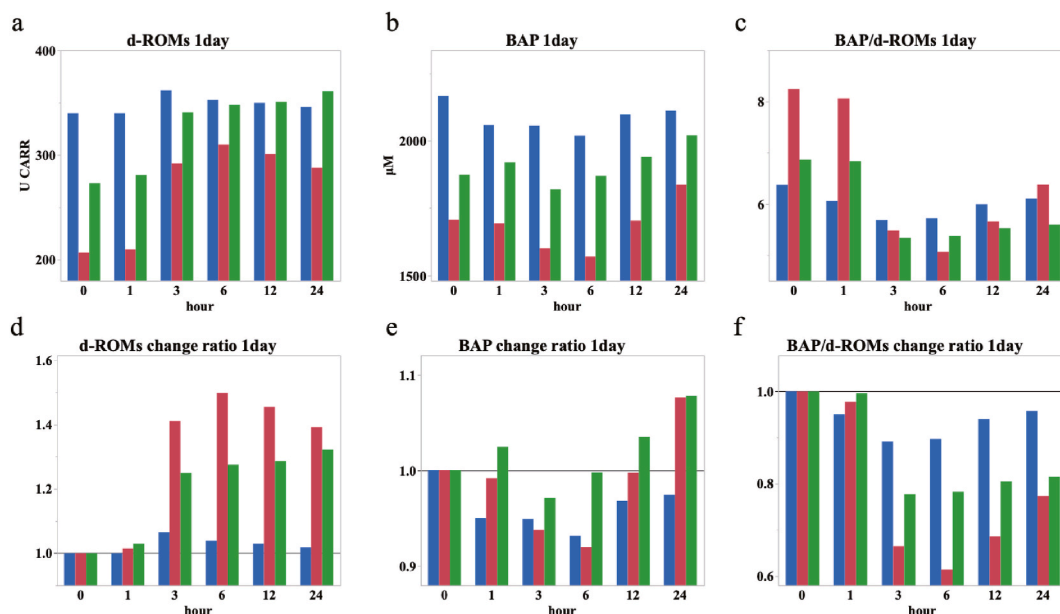

Fig. 2. d-ROMs, BAP, and BAP/d-ROMs values until 24 h
(a–f) Bar chart indicating the absolute values (a–c) and amount of change ratio relative to the initial value (d–f) of d-ROMs (a, d), BAP (b, e), and BAP/d-ROMs ratio (c, f). Time scale: 0, 1, 3, 6, 12, and 24 h; Blue: No. 1, red: No. 2, and green: No. 3.

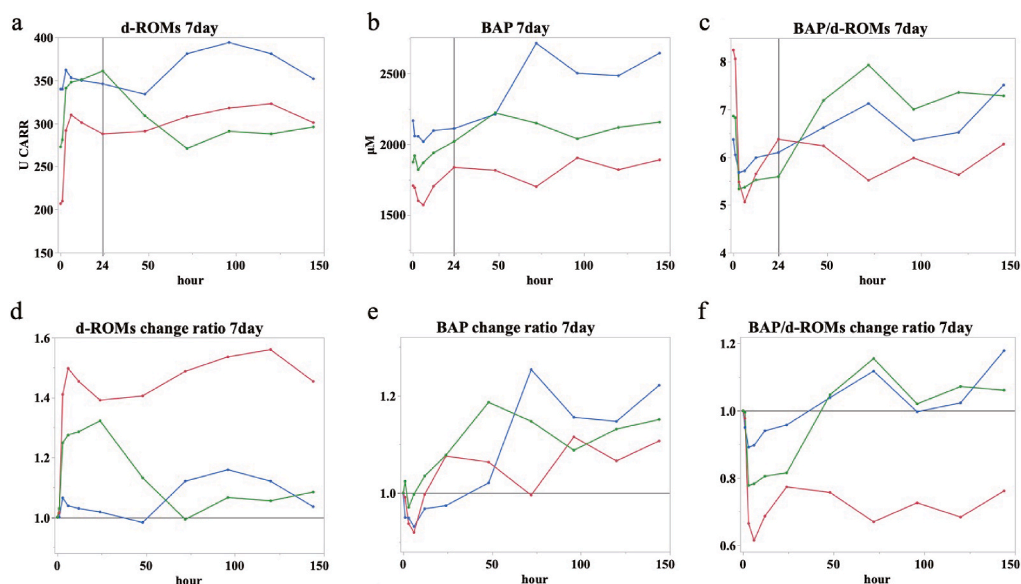

Fig. 3. d-ROMs, BAP, and BAP/d-ROMs values until 7 days
(a–f) Line graph indicating the absolute values (a–c) and amount of change ratio relative to the initial value (d–f) of d-ROMs (a, d), BAP (b, e), and BAP/d-ROMs ratio (c, f). Time scale: 0, 1, 3, 6, 12, and 24 h, and 2, 3, 4, 5, 6, and 7 days. Blue: No. 1, red: No. 2, and green: No. 3.

(Fig. 4c).

4. Discussion

After blood collection, the measured d-ROMs and BAP changed significantly over time. The d-ROMs value was stable for 1 h and increased thereafter. No major changes were observed in the BAP within 24 h; however, an increasing trend was found thereafter. Changes in blood potassium and glucose levels over time were consistent with previous reports, indicating that centrifugation within 1 h is important in studies involving the d-ROMs and BAP tests.

The d-ROMs and BAP tests have high reproducibility and statistical linearity was observed in the test values of samples with diluted concentrations (Fig. 1b–e), meaning that these tests are highly accurate. The significant measurement ranges were 40–1000 U CARR for d-ROMs and 500–6000 μM for BAP. These ranges were suggested by the manufacturer and have been validated in previous studies; therefore, they provide important thresholds for interpreting the test results [14,15]. In fact, in our previous reports, no values above the significant range were detected, regardless of age or gender [16]. In summary, the high reproducibility and linearity observed within these ranges confirm the reliability of the d-ROMs and BAP tests for clinical and research applications.

In clinical research, oxidative stress is often evaluated by either the internal oxidants or the in vivo antioxidant capacity alone. However, since oxidative stress is a balance between oxidants and antioxidant capacity, it can only be accurately evaluated by analyzing both parameters on the same basis. The BAP/d-ROMs ratio offers additional clinical significance by providing a more comprehensive picture of the oxidative balance in the body. A higher ratio indicates a robust antioxidant defense relative to the oxidative stress, while a lower ratio suggests a potential risk for oxidative damage. This ratio can be particularly useful in monitoring patients with conditions such as metabolic syndrome [5], MELAS (Mitochondrial myopathy , encephalopathy , lactic acidosis , stroke-like episodes) [17], and cancer [7], where oxidative stress plays a crucial role.

Three hours after blood collection, d-ROMs values showed an increasing trend over time (Fig. 2a–d, 3a, d). BAP values increased gradually over time from 1 day after blood collection (Fig. 2b–e, 3b, e). The blood glucose levels decreased with increasing storage time (Fig. 4b). The increase in d-ROMs values up to 24 h may be caused by an increase in oxidized metabolites in the blood due to factors, such as blood cell metabolism and hypoglycemia (Fig. 2a and d). R. Lupoli et al. reported that postoperative hypoglycemia is associated with vascular endothelial cell dysfunction and oxidative stress [18]. Increased hypoglycemia may be related to the d-ROMs levels remaining high after 24 h (Fig. 3a, d, 4b). In addition, the rapid increase in the BAP values after 24 h may have been caused by hemolysis. Furthermore, elevated potassium levels reflects hemolysis [19], and photographs of the blood samples showed that the degree of hemolysis increased proportionally with blood potassium level (Fig. 4a–c).

Through hemolysis, various substances are excreted from red blood cells into plasma. Among them, an increase in bilirubin, which has strong antioxidant properties [20], is thought to be responsible for elevated d-BAP levels. Hemoglobin and hem iron released into the blood via hemolysis affect oxidative stress [21]. Lactate dehydrogenase, which is elevated in the blood due to hemolysis, has also been implicated in causing oxidative stress [22], and these substances may have an effect in testing for oxidative stress.

Previous reports and our findings show that when performing oxidative stress analysis using samples with unseparated blood cell components, even with the addition of heparin and storage at 4 °C, oxidized metabolites may increase because of increased cellular

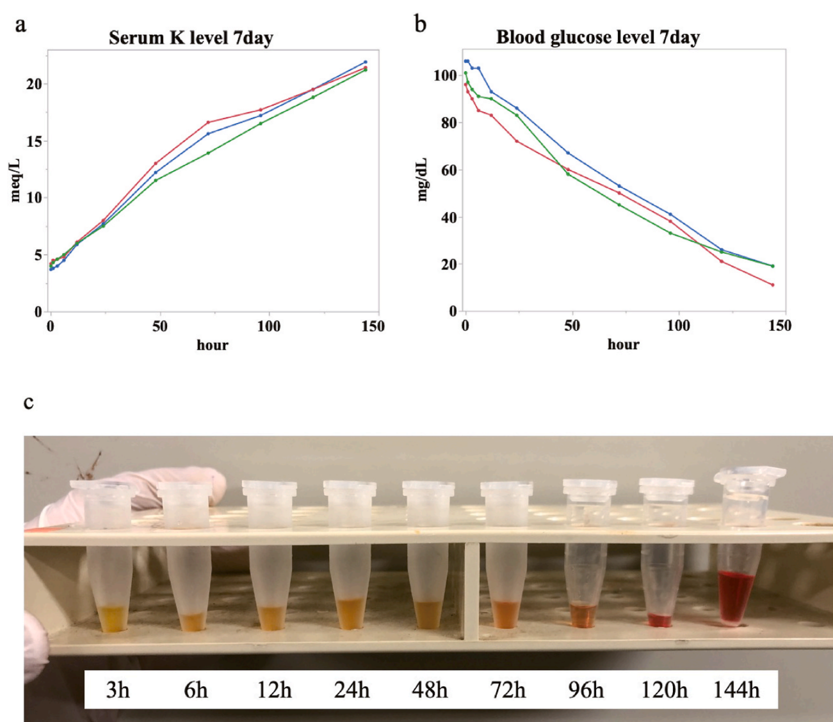

Fig. 4. Blood potassium (K) concentration and blood glucose levels and changes in appearance (a, b) Line graph indicating chronological changes in potassium (a) and glucose (b) level in blood over 7 days. Time scale: 0, 1, 3, 6, 12, and 24 h, and 2, 3, 4, 5, 6, and 7 days. Blue: No. 1, red: No. 2, and green: No. 3. (c) Representative photo of plasma samples at each measurement time.

metabolism and an increase in reducing power due to the progression of hemolysis. Thus, the analysis results were highly dependent on the storage period and unstable. A study on labor showed that this test is highly reproducible [23], proving that the changes in the test values in our study may be attributed to time. However, it is important to recognize the limitations of this study, including the lack of bilirubin concentration measurements and the limited results to adult males from the same age group.

5. Conclusion

Assessment of oxidative stress in clinical practice is essential for the diagnosis and monitoring of various conditions. This study revealed that storage of plasma samples at 4 °C significantly affected test results over time. We recommend separating plasma or serum within 1 h of blood collection to accurately assess oxidative stress.

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Disclosure of interest

The authors report no conflict of interest.

CRediT authorship contribution statement

Tomoo Yuba: Writing – original draft, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Yoshihisa Koyama:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Chiyo Ootaki:** Writing – original draft, Validation, Resources, Project administration, Formal analysis. **Yuji Fujino:** Writing – review & editing, Supervision, Project administration. **Shoichi Shimada:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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