Examination of central nervous system by functional observation battery after massive intravenous infusion of carbon monoxide-bound and oxygen-bound hemoglobin vesicles in rats

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ABSTRACT

Carbon monoxide (CO) is known as a toxic gas inducing “CO poisoning”, which acutely affects the central nervous system (CNS) and which persistently affects brain functions depending on the exposure time and CO concentration. By contrast, in pathological rodent models, intravenous infusion of CO-bound hemoglobin vesicles (CO-HbV) has shown various beneficial effects such as anti-oxidative and anti-inflammatory reactions. This study assessed effects of CO-HbV infusion on CNS using a functional observation battery, sensory reflexes, grip strength, and landing foot splay measurements. The test fluids were CO-HbV and O2-bound HbV (O2-HbV) suspended in saline ([Hb] = 10 g/dL), and saline alone for comparison. The rats received either 16 or 32 mL/kg of fluid intravenously at 1.5 mL/min/kg. Observations were made before infusion, and at 5 min, 4, 8, 24, 48 and 72 h after infusion. Massive doses of 16 and 32 mL/kg respectively corresponded to about 29 and 57% of the whole circulating blood volume (56 mL/kg). No toxicological effect was observed in any measurement item for any group in comparison to the control saline infusion group. Histopathological examination of hippocampal tissue at 14 days after infusion showed the number of necrotic cells to be minimal. Results obtained from rats in this experiment suggest that the massive intravenous infusion of CO-HbV yields beneficial anti-oxidative and anti-inflammatory effects without showing CO-poisoning-related symptoms of CNS damage.

1. Introduction

Hemoglobin vesicles (HbV) have been developed as artificial oxygen carriers that are useful as a transfusion alternative. Purified human ferrous Hb is concentrated to about 40 g/dL and encapsulated in submicrometer liposomes (230–280 nm) and suspended in a physiological saline solution. The in vivo safety and efficacy of HbV in animal models have been confirmed by academic consortia (Sakai, 2017; Yuki et al., 2021; Kohno et al., 2017; Takase et al., 2021). Even though the present established blood donation and transfusion system has contributed considerably to the present medical care system, various limitations exist, mainly the short preservation period for blood, 3–6 weeks in a refrigerator, which limits logistics and stockpiling, but with the possibility of infectious diseases and blood type mismatching, and with the decreasing number of blood donations because of a decline in young donors in aging societies and during the COVID-19 pandemic (Mili et al., 2021; Stanworth et al., 2020). Given those circumstances, realization of a blood substitute is expected to alleviate such shortcomings and constraints (Chang et al., 2022). Various kinds of chemically modified Hb-based oxygen carriers as a blood substitute have been developed, but no material is clinically available yet except some countries because of the side effects of cell-free Hb (Jahr et al., 2021; Alayash, 2019). HbV encapsulates Hb and shields such side effects of Hb. Before starting phase 1 clinical trials of oxygen-bound HbV (O2-HbV), it was necessary to undertake toxicological examinations including assessment of neurological effects by a functional observation battery (FOB) using rodents performed under the guidance of Good Laboratory Practice (GLP) (Mead et al., 2016; Sakai et al., 2022). The results, which are disclosed in this paper, showed no toxicological symptoms as we expected, and...
encouraged us to initiate a phase 1 clinical study.

In addition to their promising role as O₂ carriers, HbVs have been proven to be effective as carbon monoxide (CO) carriers showing antioxidative and anti-inflammatory reactions (Nagao et al., 2014, 2016; Sakai et al., 2009; Taguchi et al., 2018, 2021; Watabe et al., 2022; Rikihisa et al., 2022). The affinity of CO to Hb is about 200 times higher than that of O₂. Therefore, CO binds more strongly and stably to Hb than O₂ does. However, once CO-bound HbV was injected into blood circulation, CO is released gradually. It is replaced with O₂ as shown in Eq. (1).

\[
\text{CO-HbV} + O_2 \rightleftharpoons O_2\text{-HbV} + \text{CO}
\]  (1)

This exchange reaction can be facilitated toward the right side because of the abundance of O₂ on the left side (in blood), which moves the equilibrium of Eq. (1) to the right. After showing beneficial effects, probably interacting with the enzymatic heme proteins that produce reactive oxygen species, CO appears among the exhaled gases (Sakai et al., 2009).

Despite the beneficial effects of CO to Hb (Otterbein et al., 2000; Ryter and Otterbein, 2004; Zuckerbraun et al., 2005), one concern is whether the released CO might cause so-called “CO poisoning” (Prockop and Chichkova, 2007; Roderique et al., 2015) entailing the acute minor toxicities as behavioral abnormalities and physical strength change have been reported (Garay et al., 2017; Mattsson et al., 1990; Sills et al., 2000). The main purpose of this study is clarification of the absence of acute behavioral toxicity of CO released from infused CO-HbV on CNS in rats by FOB and comparison of the results with those infused with O₂-HbV.

2. Materials and methods

2.1. Preparation of HbV

From unused packed red blood cell (RBC) concentrate provided by the Japanese Red Cross (Tokyo, Japan), Hb was purified (Sakai et al., 2002). After rinsing RBC with saline by centrifugation, it was hemolyzed by adding pure water and Hb was isolated from stroma by ultrafiltration (cut-off Mw. 1,000 kDa; EMD Millipore Corp., Billerica, MA, USA). Then Hb was carboxylated by exposing to CO gas flow. The resulting carbonyl hemoglobin (HbCO) solution was heated at 60 °C for 12 h for pasteurization. The concomitant proteins other than HbCO and potential viruses were denatured to become insoluble, and easily removed using gentle centrifugation (2000×g, 30 min). The supernatant HbCO solution was then nano-filtered for virus removal using Viresolve (EMD Millipore Corp.), dialyzed, and concentrated to a concentration higher than 40 g/dl using ultrafiltration (cut-off Mw. 8 kDa, EMD Millipore Corp.).

Encapsulation of HbCO solution in liposomes was performed using a kneading method with a rotation-revolution mixer (ARE-500; Thinky Corp., Tokyo, Japan) under sterile conditions (Kure and Sakai, 2021). The lipid membrane of the liposome comprised 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, cholesterol, 1,5-O-dihexadecyl-N\text{-}scuccinyl-L-glutamate, and 1,2-diestearoyl-sn-glycero-3-phosphatidylethanolamine-N\text{-}poly(ethylene glycol) at the molar ratio of 5:4:0.9:0.03. CO-HbV was suspended in physiological saline solution at the HbCO concentration of 10 g/dl. Equimolar pyridoxal 5°-phosphate to HbCO was co-encapsulated. After CO-HbV was exposed to visible light under O₂ flow to convert HbCO to HbO₂, it was then exposed to N₂ flow to convert HbO₂ to deoxyHb in liposomes for long-term storage. The averaged particle diameter was measured as 230–280 nm using a light scattering method (nanoparticle analyzer, SZ-100; Horiba Ltd., Kyoto, Japan).

Just before an animal experiment the stored deoxygenated HbV was exposed to CO gas to prepare CO-HbV. Deoxygenated HbV was easily converted to O₂-HbV by exposure to air. Just before infusion, CO-HbV and O₂-HbV were filtrated with a disposable filter with 5.0 μm pore size (Sartorius AG).

2.2. Animals and experiment conditions

The Institutional Animal Care and Use Committee of Nihon Bio-research Inc. approved the entire experimental protocol (Study No. 360364, 410165). The protocol complies with the Basic Guidelines for the Use of Experimental Animals in Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare, Japan (Notification No. 0601001 of the Science Bureau, Japanese Ministry of Health, Labour and Welfare, Japan, June 1, 2006) in accordance with the Declaration of Helsinki, and the Guidelines for Management and Welfare of Experimental Animals (Nihon BioResearch Inc., April 2, 2007). The experiment was conducted in compliance with Ministerial Ordinance on Good Laboratory Practice for Nonclinical Safety Studies of Drugs (Ordinance of the Ministry of Health and Welfare No. 21 of March 26, 1997), the guideline for “Safety Pharmacology Studies for Human Pharmaceuticals” (ICH Q7A) (June 21, 2001, Pharmaceutical and Medical Safety Bureau, Evaluation and Licensing Division Notification No. 902), and the US National Research Council’s Guide for the Care and Use of Laboratory Animals.

The Crl:CD (SD) rats (male, 7 weeks old, 220–270 g b.w.) were purchased from The Jackson Laboratory Japan, Inc. (Yokohama, Japan). The tails and extremities of rats were marked with a green animal marker on the day of purchase. After 5 days of quarantine and 2 days of acclimation, the healthy rats were grouped. The grouping was performed one day before the experiment using a computer program (IBUKI; Nihon Bio-research Inc.) aiming at homogeneous distribution of body weight. The remaining animals were euthanized by bleeding from the abdominal aorta under isoflurane anesthesia.

The rats were housed in stainless cages (240 W × 380 D × 200 H mm) individually in air-conditioned rooms (temperature: 20.0–26.0 °C; humidity: 40–70%RH) with a 12-h dark/ light cycle and 12 times/h ventilation. The rats were provided ad libitum access to food and water.

Each rat was fixed in a Ballman cage. The test fluid (CO-HbV, O₂-HbV or saline) was infused into a tail vein at dose rates of 16 or 32 mL/kg through an indwelling needle (G24; Terumo Corp., Tokyo, Japan) connected with an extending tube (Terumo Corp.) and a polypropylene disposable syringe (Terumo Corp.). The syringe was set on a constant infusion pump (SP-110, SP-115 or SP-300; JMS Co., Ltd., Hiroshima, Japan).
Japan. The infusion rate was 1.5 mL/kg/min. The infusion was performed from 8 a.m. until noon.

We estimated that the dosage of O₂-HbV as a transfusion alternative in a clinical situation would be approximately 800 mL, which corresponds to 2 units of blood, for a patient with roughly 50 kg body weight. In this situation, the dosage is calculated as 16 mL/kg body weight. For a set of safety evaluation of O₂-HbV, a double dosage of 32 mL/kg is also tested. In this study we fixed same dosages for CO-HbV to compare the results with those of O₂-HbV.

The experiments of O₂-HbV (16 and 32 mL/kg, n = 6 each) and CO-HbV (16 and 32 mL/kg, n = 6 each) were conducted separately in different years (O₂-HbV, in 2017; CO-HbV, in 2022). These two sets of experiments had individual control saline injection group (32 mL/kg i.v., n = 6). Accordingly, the two entire studies included six groups.

2.3. Functional observation battery (FOB)

The following three categories of observations were conducted one day before injection, and at 5 min, and 4, 8, 24, 48, and 72 h after injection. In each observation, the rat group was blinded to examiners.

1) Home cage observations

Posture, palpebral closure, stereotypical behaviors (excessive grooming, repetitive circling, biting behavior), clonic convulsions, and tonic convulsions were observed when the animals were in cages.

2) Hand-held observations

Ease of removal from a cage, ease of handling, muscle tone, fur conditions, mucous membranes, lacrimation, salvation, piloerection, pupil size, and respiration were observed when the animals were removed from the cage and held by hand.

3) Open field observations

Frequencies of urination, defecation, rearing, and grooming during a 2-min period were observed when the animal was released in an open field. Moreover, gait, palpebral closure, consciousness, behavioral abnormality, and righting reflex were observed in an open field.

2.4. Sensory reflexes

After FOB, pupillary reflex, approaching behavior, response to touch, auditory reflex and pain reflex were observed when the animals were put on a table.

2.5. Grip strength

The measurements of grip strength of the animals were performed one day before injection, 5 min, 24 and 72 h after injection. A force gauge (Aikoh Engineering Co., Ltd., Osaka, Japan; for CO-HbV study) and another force gauge (San Diego Instruments Inc., San Diego, USA; for O₂-HbV study) were used to measure forefoot and hindfoot grip strength (g) five times. Of the five values, the highest and the lowest values were omitted; the remaining three values were averaged and used to express mean ± S.D.

2.6. Landing foot splay

After the bottom of hindfoot was painted with ink, the animal was dropped freely from 30 cm height. The length of the landing foot splay was measured five times using a digital caliper (CD-15; Mitutoyo Corp., Kawasaki, Japan). Of the five values, the highest and lowest values were omitted. The remaining three values were averaged and used to express mean ± S.D.

2.7. Body temperature and body weight

Body temperature was measured during the above hand-held observation using a thermometer (BDT-100; Bio Research Center Co. Ltd., Nagoya, Japan) equipped with a sensor (RET-2; Bio Research Center Co. Ltd.). The sensor was inserted into the rectum. Body weight was measured before and 72 h after infusion of the test material.

2.8. Histopathological examination of brain

After the final measurements at 72 h, three rats of each group (n = 3) were randomly selected and caged for an additional 11 days (completely 14 days after infusion). Other rats were euthanized by bleeding from the abdominal aorta under isoflurane anesthesia. On day 14, the rats were euthanized by bleeding from the abdominal aorta under isoflurane anesthesia while perfusing saline solution from the left ventricle with subsequent perfusing 10% neutral formalin solution (Sigma-Aldrich Japan K.K., Tokyo, Japan) to fix the tissue (Okuda and Sakai, 2022). The brains were removed and stored in the 10% neutral buffered formalin solution.

The paraffin sections of the hippocampus were stained with hematoxylin/eosin (HE) (Nara-byouri Laboratory Co., Ltd., Nara, Japan) to assess the cellular structure. All cells (about 1,000–2,000 cells) in the hippocampal area on the right or left side of these specimens were evaluated to ascertain the ratio of normal to necrotic cells. Necrotic cells were defined as those which did not retain their cell shape and the nuclei of which could not be identified. The finesse of brain perfusion to fix the brain tissues at sacrificing and the method of microtome section might also potentially affect the cell shape. Such factitiously abnormal cells would be highly likely to be counted as necrotic cells.

2.9. Statistical analysis

Results of FOB and sensory reflexes were not analyzed statistically. Grip strength, landing foot splay, and body temperature were expressed as mean ± standard deviation. Tests of statistical significance were performed for body temperature and landing foot splay between the groups using Bartlett tests to examine the homogeneity of variance, followed by Dunnett tests. P values of 0.05 or less were inferred as indicating significance.

3. Results

3.1. Functional observation battery (FOB)

As for the home cage observations, the results of posture, palpebral closure, stereotypical behaviors (excessive grooming, repetitive circling, biting behavior), clonic convulsions, and tonic convulsions are presented in Table 1. Grades of the findings for each measurement are listed as footnotes. No abnormal behavior for any group, including CO-HbV (32 mL/kg), was observed at any time point.

As for hand-held observations, the results of ease of removal from a cage, ease of handling, muscle tone, fur conditions, mucous membranes, lacrimation, salvation, piloerection, pupil size, and respiration are presented in Table 2. The grades of the findings for each measurement are listed as footnotes. No abnormal behavior for any group, including CO-HbV (32 mL/kg), was observed at any time point.

Frequencies of urination (Supplemental File Table S1), defecation (Supplemental File Table S2), rearing (Supplemental File Table S3), and grooming (Supplemental File Table S4) during a 2-min period in individual rats of CO-HbV and O₂-HbV groups showed individual differences and fluctuations, but no marked difference to the control saline groups was observed.

The results obtained for gait, palpebral closure, consciousness, behavioral abnormality, and righting reflex in an open field are presented in Table 3. The grades of the findings for each measurement are listed as...
72 h after injection. Identical for all rats in each group before injection, and at 5 min, 4, 8, 24, 48 and 72 h after injection. Presented in Table 4. The grades of the findings were identical for all rats in each group before injection, and at 5 min, 4, 8, 24, 48 and 72 h after injection.

Table 1
Observations of rats in cages. Grades of the findings were identical for all rats in each group before injection, and at 5 min, 4, 8, 24, 48 and 72 h after injection.

| Endpoints | CO-HbV (mL/kg) | Control saline 32 mL/kg | O2-HbV (mL/kg) | Control saline 32 mL/kg |
|-----------|----------------|-------------------------|----------------|------------------------|
| Posture   | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Palpebral closure | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Excessive grooming | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Repetitive circling | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Biting behavior | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Clonic convulsions | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Tonic convulsions | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |

* Findings were graded as presented below. Posture: prone or recumbent position (1), sitting normally (2), running or moving about (3), and jumping (4); Palpebral closure: eyes open normally (1), eyelids half-closed (2), and eyelids closed (3); Excessive grooming: not observed (1) and observed (2); Repetitive circling: not observed (1) and observed (2); Biting behavior: not observed (1) and observed (2); Clonic convulsions: not observed (1), jaw convulsions (2), and tremor (3).

Table 2
Hand-held observation of rats on observer’s palm. Grades of the findings were identical for all rats in each group before injection, and at 5 min, 4, 8, 24, 48 and 72 h after injection.

| Endpoints | CO-HbV (mL/kg) | Control saline 32 mL/kg | O2-HbV (mL/kg) | Control saline 32 mL/kg |
|-----------|----------------|-------------------------|----------------|------------------------|
| Ease of removal from cage | 2 2 2           | 2 2 2                   | 2 2 2          | 2 2 2                  |
| Ease of handling | 2 2 2           | 2 2 2                   | 2 2 2          | 2 2 2                  |
| Muscle tone | 2 2 2           | 2 2 2                   | 2 2 2          | 2 2 2                  |
| Fur condition | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Mucous membrane | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Lacrimation | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Salivation | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Piloerection | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Pupil size | 2 2 2           | 2 2 2                   | 2 2 2          | 2 2 2                  |
| Respiration | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |

* Findings were graded as presented below. Ease of removal from cage: docile and allowing itself to be handled (1), rearing or cowering (2, normal score), and running about and difficult to catch (3); Ease of handling: docile and allowing itself to be handled (1), struggling slightly or vocalizing (2, normal score), and struggling and trying to bite observer’s hand (3); Muscle tone: depressed (1), normal (2), and increased (3); Fur conditions: normal (1), slightly soiled (2), and markedly soiled (3); Mucous membranes: normal (1), brown (2), hemorrhage (3), and swelling (4); Lacrimation: none (1), mild (2), and marked (3); Salivation: none (1), mild (2), and marked (3); Piloerection: none (1), mild (2), and marked (3); Pupil size: mydriasis (1), normal (2), and miosis (3); Respiration: normal (1), bradypnea (2), and dyspnea (3).

Table 3
Observations of rats in an open field. Grades of the findings were identical for all rats in each group before injection, and at 5 min, 4, 8, 24, 48 and 72 h after injection.

| Endpoints | CO-HbV (mL/kg) | Control saline 32 mL/kg | O2-HbV (mL/kg) | Control saline 32 mL/kg |
|-----------|----------------|-------------------------|----------------|------------------------|
| Gait      | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Palpebral closure | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Consciousness | 2 2 2           | 2 2 2                   | 2 2 2          | 2 2 2                  |
| Behavioral abnormalities | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |
| Righting reflex | 1 1 1           | 1 1 1                   | 1 1 1          | 1 1 1                  |

* Findings were graded as presented below. Gait: normal (1), unmoving (2), staggering (3), hind-limbs extended and dragged (4), all fours extended (5); Palpebral closure: eyelids open normally (1), eyelids half-closed (2), and eyelids closed (3); Consciousness: comatose, with no response (1), exploring behavior (2, normal score), and excited and moving spasmodically (3); Behavioral abnormalities: not observed (1), Straub's reaction (2), moving backward (3), writhing (4), and self-biting (5); Righting reflex: righting itself immediately (1, normal score), requiring 3 s or longer to right itself (2), and unable to right itself (3).

3.2. Grip strength

Results of forefoot grip strength (Fig. 1A) showed no significant difference among groups at any time point. Results of hindfoot grip strength (Fig. 1B) showed no difference among CO-HbV groups (16 and 32 mL/kg) and the saline control group. The O2-HbV groups and the saline control group showed lower values until 24 h because a different force gauge was used. In that experiment, the measurements were also conducted at other time points (4, 8, and 48 h). Such frequent measurements might be so stressful for rats that the muscle tone should be increased at 72 h. However, it is important to note that no significant difference was found among groups at any time point.

3.3. Landing foot splay

The lengths of landing foot splay are presented in Fig. 1C. No significant difference was found among groups, including CO-HbV (32 mL/kg), at any time point.

3.4. Body temperature and body weight

The changes in body temperature are presented in Fig. 1D. For all the groups, including the saline control groups, the body temperature increased only at 5 min after injection. Probably, the rats were excited soon after holding in a Ballman cage and injection of test fluids. The temporary increase in body temperature would also be related to the acute volume overload, which stimulates the sympathetic nerve system. However, no significant difference was found among groups at any time point.

The body weight was around 330–340 g before and 72 h after infusion of CO- or O2-bound HbV at 16 or 32 mL/kg. No significant difference was found among groups (Supplemental File Figure S1). Judging from frequencies of urination and defecation, eating habits were apparently unaffected.

3.5. Histopathological examination of brain

At 14 days after injection of CO-HbV and control saline solution, hippocampal tissue samples (HE stain) were examined (n = 3) (Fig. 2). Very small amounts of morphologically abnormal cells, seemingly necrotic cells, were detected in all groups. The CO-HbV (32 mL/kg)
CO-HbV does not affect CNS in this experiment model. The responses of massive infusion of CO-releasing HbV in rats by FOB and physical examination were not observed (1), approaching and suffering stimulus (2), reacting to stimulus, including vocalizing (3), and jumping at or biting at stimulus (4); Response to touch: no response (1), hesitating at stimulus or moving ears (2), and jumping at and trying to bite at the source of sound (3); Pain reflex: not observed (1), slowly looking back or slowly moving forward to escape from stimulus (2), quickly moving forward to escape from stimulus or biting at it immediately after looking back (3), jumping forward to escape stimulus (4), and loudly vocalizing and biting at stimulus after suddenly looking back (5).

The dosages of 16 and 32 mL/kg respectively correspond to about 10 g/dL, should theoretically increase the oxygen carrying capacity of 32 mL/kg infusion of CO-HbV does not affect CNS in this experiment model. The responses closely resemble those when infused with 32 mL/kg O2-HbV.

Table 4

| Endpoints          | Grades of findings | O2-HbV (mL/kg) | Control saline (mL/kg) | O2-HbV (mL/kg) | Control saline (mL/kg) |
|--------------------|--------------------|----------------|-----------------------|----------------|------------------------|
|                    |                    | 16 mL/kg       | 32 mL/kg              | 16 mL/kg       | 32 mL/kg               |
| Pupillary reflex   | 2                   | 2              | 2                     | 2              | 2                      |
| Approaching behavior | 2                   | 2              | 2                     | 2              | 2                      |
| Response to touch  | 2                   | 2              | 2                     | 2              | 2                      |
| Auditory reflex    | 2                   | 2              | 2                     | 2              | 2                      |
| Pain reflex        | 3                   | 3              | 3                     | 3              | 3                      |

*Findings were graded as presented below. Pupillary reflex: pupils completely dilated (1), normal pupillary contraction observed (2), and pupils completely contracted (3); Approaching behavior: not observed (1), approaching and snuffing stimulus (2), reacting to stimulus, including vocalizing (3), and jumping at or biting at stimulus (4); Response to touch: no response (1), looking back and leaving stimulus (2), reacting to stimulus, including vocalizing (3), and jumping at or biting at stimulus (4); Auditory reflex: not observed (1), hesitating at stimulus or moving ears (2), and jumping at and trying to bite at the source of sound (3); Pain reflex: not observed (1), slowly looking back or slowly moving forward to escape from stimulus (2), quickly moving forward to escape from stimulus or biting at it immediately after looking back (3), jumping forward to escape stimulus (4), and loudly vocalizing and biting at stimulus after suddenly looking back (5).

Various CO-releasing materials intended for *in vivo* use have been reported, including CO-metal complex, synthetic organic molecules, HbV, and RBCs, and others for aiming at anti-inflammatory and anti-oxidative effects (Sakai et al., 2009; Cabrales et al., 2007; García-Gallego and Bernardes, 2014; Motterlini and Foresti, 2017; Ogaki et al., 2013; Popova et al., 2018; Misra et al., 2011; Yan et al., 2019; Braud et al., 2018; Pathak et al., 2022). However, these studies did not thoroughly scrutinize the potential toxicity of released CO especially on neurological effect. This report is the first to describe a study of the effects of massive infusion of CO-releasing HbV in rats by FOB and physical strength measurement, revealing that at least 32 mL/kg infusion of CO-HbV does not affect CNS in this experiment model. The responses closely resemble those when infused with 32 mL/kg O2-HbV.

The dosages of 16 and 32 mL/kg respectively correspond to about 29% and 57% of the circulating whole blood volume. Intravenous infusion of CO-HbV, of which [Hb] = 10 g/dL, should theoretically increase the HbCO level to about 30%, and then the HbCO level should decrease gradually because of the ligand exchange reaction of Eq. (1). Most of the released CO should be eventually exhaled through the lung, according to findings of our earlier studies (Sakai et al., 2009). The preservation of the respiratory function should be important to minimize the CO gas poisoning. The level of HbCO in blood should diminish within 6 h (Sakai et al., 2009; Taguchi et al., 2018), after providing the anti-inflammatory and anti-oxidative effects. Endogenous enzymatic reactions with mitochondrial cytochrome oxidase are expected to convert CO partly to CO2 (Piantadosi, 1987; Young and Caughey, 1986).

In actuality, CO poisoning is known to induce headache, dizziness, weakness, nausea, lightheadedness, defective judgment, muscle cramping, etc., and severe acute symptoms of consciousness disturbance, fainting, respiratory failure, and hypotension. Reportedly CO gas-poisoned rats demonstrated the local myofasciculations which augmented into convulsions in 7 ± 1 min (Tolkach et al., 2016). Results of this FOB conducted from 5 min until 72 h after massive infusion of CO-HbV showed no abnormality in behavior or sensory reactivity (Tables 1–4, Supplementary File Tables S1–S4). The results also clarified that infusion of CO-HbV does not induce weakness, as judged from grip strength and landing foot splay findings (Fig. 1). Results of earlier studies have demonstrated that respiratory and cardiovascular functions were maintained after bolus infusion of CO-HbV in mice. Moreover, plasma clinical chemistry showed that hepatic and renal functions were preserved (Nagao et al., 2016). Reduced respiratory and cardiovascular functions induced by hemorrhagic shock were recovered by resuscitation with CO-bound HbV and RBC (Sakai et al., 2009; Cabrales et al., 2007; Ogaki et al., 2013). Overall, the results obtained from our experimental setting of 32 mL/kg dosing indicate that the massive intravenous infusion of CO-HbV to rats does not induce “CO poisoning” in the acute phase.

After showing acute toxicities, “CO poisoning” is known to induce pathological changes slowly in the brain hippocampus leading to symptoms of neurological damage, showing cognitive and memory dysfunctions (Ernst and Zibarak, 1998; Shen et al., 2016; Bi et al., 2017; Piantadosi et al., 1997). Our earlier study using CO poisoned rats prepared by CO inhalation demonstrated that pathological changes in the hippocampus were prominent after 14 days (Okuda and Sakai, 2022). This study confirmed that the pathological changes in the hippocampus were minimal at 14 days after intravenous massive infusion of CO-HbV (Fig. 2). This finding is plausible because rats with no acute symptoms would not be expected to show delayed symptoms.

The question arises of why a massive intravenous infusion of CO-HbV did not induce CO poisoning-like symptoms. Earlier reports have described that inhaled CO is more toxic than HbCO or CO gas infused by intraperitoneal injection (Goldbaum et al., 1975, 1976; Young and Caughey, 1990). Even though CO binds about 200 times more tightly to Hb than does O2, the binding rate constant of CO to Hb (4.5 × 10^6 M^-1s^-1) is much lower than that of O2 (6.0 × 10^9 M^-1s^-1) (Olson, 1981). Moreover, compartmentalization of Hb in RBC further retards CO binding (Sakai et al., 2008). In our study, CO is tightly bound to HbV: less CO is expected to be transferred to tissues rapidly. However, CO inhalation would be expected to produce high tension in the alveoli and increase physically dissolved CO in the blood plasma, but not be chemically bound CO to Hb in RBC because of slow CO binding to Hb. Consequently, the amount of CO transferred directly from the lung to the brain tissue via blood plasma can be expected to increase, causing CO poisoning.

A limitation of our study is that FOB using a rat model would not be sufficient to show the absence of the symptoms of CO poisoning after infusion of CO-HbV. One should be careful to show no toxicity of CO released from CO-HbV because CO is well-known as a toxic gas. Studies of memory functions using a maze test (Tolkach et al., 2016; Piantadosi et al., 1997) or using other larger animals (Kent et al., 2010; Qingsong et al., 2013) would be necessary to confirm the neurological safety of CO-HbV.

Regarding O2-HbV as a blood substitute (artificial oxygen carrier), we completed GLP preclinical studies including FOB as shown in this study, toxicological studies after bolus and repeated injections, immunogenicity testing, pharmacological testing (cardiovascular and respiratory systems), and pharmacokinetic studies using rodents and beagles (Sakai et al., 2022), and then conducted Phase 1 clinical study of healthy male adults (Azuma et al., 2022). The results encourage us to continue further development. Following the mode used for O2-HbV, we continue the development of CO-HbV as a new category of CO-carrier for anti-inflammatory and anti-oxidative agents (Rikihisa et al., 2022; Taguchi et al., 2017).
5. Conclusion

FOB and physical strength measurements clarified that intravenous massive infusion of CO-HbV does not induce symptoms of “CO poisoning” at the dose of 32 mL/kg in a rat model. The results are almost identical to those obtained for O₂-HbV infusion. Many earlier studies have demonstrated beneficial effects of the released CO. However, further study is expected to be necessary to confirm the preservation of memory function in rats and other larger animal models to dispel the basic prejudice against CO as a toxic gas.
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CRediT authorship contribution statement

Hiromi Sakai: Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Shunichi Yasuda: Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Supervision, Project administration.

Chie Okuda: Investigation, Formal analysis, Visualization.

Tetsuya Yamada: Investigation, Formal analysis.

Keita Owaki: Investigation, Formal analysis.

Yoji Miwa: Methodology, Validation, Investigation.

Declaration of competing interest

Owaki:

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