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# Automatic labeling method for injectable $^{15}$O-oxygen using hemoglobin-containing liposome vesicles and its application for measurement of brain oxygen consumption by PET

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**Abstract**

**Introduction:** The aim of this study was to develop an injectable $^{15}$O-$O_2$ system using hemoglobin-containing vesicles (HbV), a type of artificial red blood cell, and to investigate the feasibility of $^{15}O_2$-labeled HbV ($^{15}O_2$-HbV) to measure cerebral metabolic rate of oxygen ($CMRO_2$) in rats.

**Methods:** The direct bubbling method was combined with vortexing to enhance labeling efficiency of HbV with $^{15}$O-$O_2$ gas. L-Cysteine was added as a reductant to protect hemoglobin molecules in HbV from oxidation at different concentrations, and labeling efficiencies were also compared. Measurement of cerebral blood flow (CBF) and $CMRO_2$ in five normal rats was performed using a small animal PET scanner after the injection of $H_2^{15}O$ and $^{15}O_2$-HbV to evaluate the precision of hemodynamic parameters quantitatively.

**Results:** The labeling efficiency of HbV was significantly increased when vortexing and bubbling were combined compared with the simple bubbling method ($P<.05$). The most efficient method for labeling was bubbling of $^{15}$O-$O_2$ combined with vortexing and the addition of 2.8 mM L-cysteine in HbV solution. The mean radioactivity of 214.4±7.8 MBq/mL HbV was obtained using this method. PET scans using $^{15}O_2$-HbV and $H_2^{15}O$ yielded a mean $CMRO_2$ value of 6.8±1.4 (mL/min per 100 g) in rats with normal CBF of 51.4±7.9 (mL/min per 100 g).

**Conclusion:** Addition of L-cysteine to HbV and simple direct bubbling of $^{15}$O-$O_2$ gas combined with vortexing was the most efficient method for preparation of $^{15}O_2$-HbV. The present injectable system using $^{15}O_2$-HbV was successfully utilized to measure $CMRO_2$ in rats, indicating that this new method could be useful for animal models to measure oxygen metabolism in the brain.


*Keywords:* Hemoglobin vesicle (HbV); Injectable $^{15}$O-$O_2$; L-cysteine; Cerebral metabolic rate of oxygen ($CMRO_2$); Positron emission tomography (PET)

## 1. Introduction

Cerebral metabolic rate of oxygen ($CMRO_2$) is measured in clinical practice using PET with inhalation of $^{15}$O-$O_2$ gas. One of the problems associated with the inhalation method is the difficulty in handling radioactive gas, causing radioactivity exposure to the patient and hospital staff. In animal models, the inhalation method is technically very difficult to implement. High radioactivity in the inhalation tube and mask may affect calculation of brain oxygen metabolism and appropriate scatter correction is required. In previous reports of an injectable $^{15}$O-$O_2$ system for small animal positron emission tomography (PET) studies, $CMRO_2$ was measured using rat blood and an artificial lung with reasonable accuracy [1,2]. However, this method is not ideal as it exposes the handler to a large amount of radioactivity and high blood flow rates are needed in the artificial lung for labeling, which may result in clotting and blood component damage as the flow tends to become turbulent at high flow rates [3]. Moreover, an extra rat has to be sacrificed for blood

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collection and preservation of collected blood is required. Therefore, developing a simpler and safer method for intravenously injectable $^{15}O$-$O_2$ would be useful for both animal experiments and human studies.

Successful labeling of whole blood by bubbling $^{15}O$-$O_2$ gas for human studies has been achieved [4]; however, direct bubbling of $^{15}O$-$O_2$ gas into the blood is reported to cause foam degeneration of plasma proteins which hinders normal functioning of red blood cells (RBCs) [1]. To prevent foam degeneration by bubbling of $^{15}O$-$O_2$ into the blood, one option is to remove protein fraction from the blood by washing the RBCs. However, it is very troublesome to wash RBCs many times, and handling during washing may affect the biologic function of the RBCs [5]. Oxygen loading and unloading characteristics of hemoglobin-containing vesicles (HbV) and their oxygen dissociation curves are almost similar to that of RBCs [6–11]. HbV, a liposome containing many hemoglobin molecules was originally developed as a RBC alternative oxygen carrier. The synthesis processes along with main chemical and rheological properties of HbV were previously reported in detail [6,8,12–14]. Recently, it was shown that HbV could combine with $^{15}O$-$O_2$ in a manner similar to RBCs and that transfusion of HbV in a hemorrhagic shock model of rat actually improves oxygen supply [15]. However, there were no studies that comprehensively evaluate the labeling method itself and the consequent evaluation of $CMRO_2$ using intravenously injectable $^{15}O$-$O_2$-labeled HbV ($^{15}O_2$-HbV). Because HbV are smaller and more stable than normal RBCs, which could better withstand any direct manipulation including direct bubbling [6–10], an injectable $^{15}O$-$O_2$ system using HbV instead of normal RBCs is desirable. Furthermore, HbV are free from plasma proteins, have a long storage time and requires no preparation besides having a reduced risk of blood-borne infections and antigen incompatibility [16].

The purpose of this study was to design and optimize an automatic system to label HbV with $^{15}O$-$O_2$ gas and establish a convenient method to measure quantitative oxygen metabolism in small animals using the single injection of $^{15}O_2$-HbV and PET. In this study, we optimized the labeling conditions to get the maximum yield in labeled HbV samples. To evaluate the efficacy of this $^{15}O_2$-HbV in vivo, $CMRO_2$ in rats was measured using a small animal PET scanner.

## 2. Materials and methods

### 2.1. HbV sample preparations

The HbV preparation was provided by Oxygenix Co. Ltd. (Tokyo, Japan). Its properties are described in Table 1. The synthesis and purification processes along with main chemical and rheological properties of HbV were previously described in detail [4,6,10–12]. Major lipid components in HbV membrane were 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPCC), cholesterol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPG) at a molar ratio of 5/5/1. Fifty-milliliter vials with 5 ml of HbV samples were used for labeling experiments. HbV samples were prepared and kept under anoxic conditions. All the chemicals used in this study were of reagent grade.

Table 1
HbV specifications

| Parameters | Values |
|---|---|
| Diameter (nm) | 277 |
| Lipid (g/dL) | 6.2 |
| Hemoglobin (g/dL) | 10.8 |
| $P^{50}$ (torr) | 23 |
| pH (at 37°C) | 7.4 |
| metHb (%) | 8.5 |
| HbCO (%) | 3.6 |

$P^{50}$, oxygen affinity; metHb, methhemoglobin; HbCO, carboxyhemoglobin.

### 2.2. $^{15}O$-$O_2$ gas production

$^{15}O$-$O_2$ gas was produced by a $^{15}N(p, n)^{15}O$ nuclear reaction with a 2.5% $O_2/N_2$ gas target at 18-μA proton current accelerated by an in-house cyclotron Eclipse HP/RD (Siemens, Knoxville, TN, USA). The bombarded target gas along with the $^{15}O$-$O_2$ gas produced in the target box was transported to the cyclotron line, and then, to our injectable $^{15}O$-$O_2$ preparation system.

### 2.3. Preparation of $^{15}O$-$O_2$-labeled HbV

The labeling system mainly consisted of a 50-ml bubbling vial containing 5 ml of HbV mounted on a vortex machine. The cyclotron line carrying target gas was connected to the vial with tubes, a three-way valve and a long needle to bubble the $^{15}O$-$O_2$ containing target gas (Fig. 1). Waste gas line was connected to the same three-way valve and a vacuum pump (KnF LAB, FTP-10 A, Asahi Techno Glass, Funabashi, Japan) to make it a closed system. To avoid any backflow during bubbling, the bubbling needle was connected to a one-way filter (Millex-FG.20 μm, Millipore Co., Bedford, MA, USA). Pressure in the bubbling line was monitored with a pressure sensor (KEYENCE, Osaka, Japan) during the entire experimental process to detect any

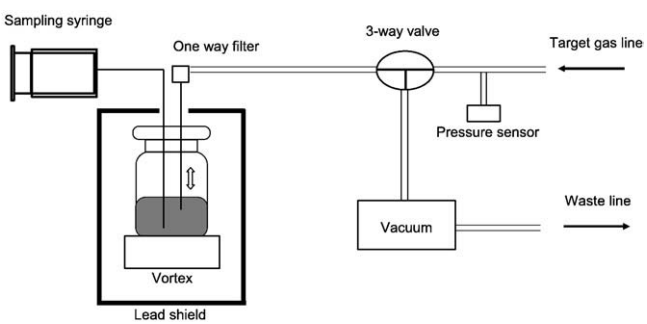


Fig. 1. Schematic diagram of the final labeling setup with lead shielding for injectable $^{15}O_2$-HbV preparation.

leakage in the system. The three-way valve connected to a motor (Dynamite AX-12, Robotis motor, Tokyo, Japan) was controlled using a computer. The labeling system was enclosed in a lead shield box (30.5×25.5×40 $cm^3$) with a uniform thickness of 5.8 cm including the base. Using this final labeling setup, HbV labeling with $^{15}O_2$ gas was carried out in the following manner. After connecting 50 ml vial with 5 ml of HbV samples to our injectable $^{15}O_2$ preparation system, vacuuming of the target line and vial was done while keeping the bubbling needle well above the HbV solution. Vacuum valve was then closed, and target gas containing $^{15}O_2$ was delivered into the vial via target line after pushing the bubbling needle inside the HbV solution. Bubbling along with vortexing was carried out for 2 min. Labeled HbV was then sampled using a sampling syringe and radioactivity is measured. The flow rate of the $^{15}O$-$O_2$ containing target gas was 200 ml/min in the target line and the radioactivity in the target gas used for HbV labeling experiments was 3700 MBq.

Using this system, the following tests were carried out to optimize the preparation of $^{15}O$-$O_2$-labeled HbV. At first, $^{15}O$-$O_2$ gas was bubbled into the HbV vial for labeling experiments. To improve the radioactivity yield in the labeled HbV, vortexing (3000 rpm) of the bubbling vial was added for 2 min. The target gas with different concentrations of oxygen (2.5%, 5% and 7.5%) was also evaluated for its efficacy and oxygen saturation (%) was measured in labeled HbV samples. Oxygen saturation in HbV was measured using a blood gas analyzer (OPTI-1, AVL Scientific Corporation, Roswell, USA). Finally, L-cysteine, a known non-enzymatic reductant [17], was added to bring down the methemoglobin level at different concentrations in HbV samples before labeling experiments. Endotoxin contamination of HbV samples was determined by the *Limulus* assay test [18] both before and after labeling experiments.

### 2.4. Animal PET Studies

Sprague–Dawley rats (250–300 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). Animal studies were conducted in accordance with institutional guideline and approved by the Animal Care Committee at the University of Fukui. Rats were starved for 6 h before PET experiments. The rats were anesthetized with the intraperitoneal injection of chloral hydrate (400 mg/kg). A small PE-50 catheter (i.d. 0.5 mm, o.d. 0.9 mm) was then inserted into the left femoral artery for sequential blood sampling. A separate line using the same tube was placed in the right femoral vein to administer the radiotracer. PET studies were performed using a PET scanner for small animals (SHR-41000; Hamamatsu Photonics, Hamamatsu, Japan). The scanner acquired 213 slices covering an axial length of 160 mm with 3D mode and achieving a resolution of approximately 2.0 mm full width at half maximum (FWHM) in the transaxial direction and 2.8 mm FWHM in the axial direction [19]. Each anesthetized rat was placed in the supine position on the scanner bed, and the limbs were fixed using surgical tape. The optimal cranial position was determined using a laser beam on a scanner. Before emission scans, a transmission scan was performed for 60 min using $^{68}Ge/^{68}Ga$.

To measure cerebral blood flow (CBF), a PET scan was conducted with intravenous administration of $H_2^{15}O$ (37 MBq, 0.5 ml) in the femoral vein using an automatic syringe pump (pump 11; Harvard Apparatus, Holliston, MA, USA) with a constant rate for 30 s. A 6-min dynamic PET scan in 3D mode was initiated at the $H_2^{15}O$ injection using list-mode acquisition. Repeated arterial blood sampling in 20–40 μl was performed manually every 5 s for the first minute, every 15 s for the next 2 min and every 30 s for the rest of the scan time with a total of 24 blood samples, which was the similar sampling method to that in the previous report [1]. The radioactivity in the blood samples thus obtained was immediately measured with a well scintillation counter (ARC 380, Aloka, Tokyo, Japan). The scintillation counter was carefully cross-calibrated with the PET scanner before the PET scans using a small cylinder pool phantom and $^{18}F^-$ solution. Fifteen minutes after the $H_2^{15}O$-PET scan, $CMRO_2$ measurement was performed with 37 MBq of $^{15}O_2$-HbV (1 ml). The injection rate for tracer, PET scan protocol and blood sampling were the same as the $H_2^{15}O$ PET study. $^{15}O_2$-HbV was injected intravenously at a constant rate for 60 s. Blood gas data were analyzed after each PET scan of $H_2^{15}O$ and $^{15}O_2$-HbV for various physiological and hematological parameters. Rat body temperature was maintained throughout the experimental process. The list-mode data acquired for initial 3 min were reconstructed using a Fourier rebinning algorithm. After rebinning the data, 3D images were reconstructed using a 2D filtered back-projection algorithm and a Hanning filter (2.8 mm) with time frames of 5 s ×12, 10 s ×6, 20 s ×3. Decay for $^{15}O$ activity was corrected to the start of the PET scan.

### 2.5. Calculation of CBF and $CMRO_2$

$H_2^{15}O$ and $^{15}O_2$ -HbV were coregistered, and using $H_2^{15}O$ images, five regions of interest (ROIs) on the whole brain were drawn at the middle level of the brain. Same set of ROIs were applied in both $H_2^{15}O$ and $^{15}O_2$ -HbV images. The time activity curve (TAC) of the whole brain was obtained by averaging ROI data from several slices. The CBF values (ml/min per 100 g) were calculated by the autoradiographic method [20,21]. A partition coefficient of 0.8 was applied for calculations [1,2].

The $CMRO_2$ values (ml/min per 100 g) were calculated based on two-compartment analysis [22]. Rate constants of $K_1$ and $k_2$ for the two-compartment model were determined by nonlinear least squares fits for the following equation (Eq. 1) using TAC from dynamic brain images and arterial input function.

$$C_O(t) = K_1 A_O(t) \otimes e^{-k2t} + V_0 A_O(t), \quad (1)$$

where $C_O(t)$ and $A_O(t)$ are the radioactivity in brain tissue and arterial blood, $V_0$ is the vascular volume and ⊗ represents convolution. The initial 3-min dynamic PET data and input function were used for the fitting. Finally, $CMRO_2$ was calculated as a product of $K_1$ and the total arterial oxygen content ($tO_2c$) as shown in Eq. 2.

$$CMRO_2 = K_1 tO_2c. \quad (2)$$

The advantage of this one-step method by Ohta et al. [22] is that $CMRO_2$ can be calculated without independent estimation of oxygen extraction fraction (OEF) because $K_1$ can be defined as CBF×OEF.

### 2.6. Data analysis and statistics

Results are expressed as the mean±S.D. For statistical data analysis, one-way analyses of variance (ANOVAs) with post hoc Scheffe's test were applied for multiple comparisons. A Student *t* test was applied for two group comparisons. Blood gas data in the PET studies were compared at two time points using paired *t* test. A *P* value of less than .05 was considered statistically significant.

## 3. Results

### 3.1. $^{15}O$-$O_2$-labeled HbV

Using bubbling-only method, the radioactivity obtained in $^{15}O_2$-labeled HbV was 28.9±9.6 MBq/mL, which increased to 79.2±11.8 MBq/mL when bubbling was combined with vortexing. The difference in the labeling efficiency of HbV between the two methods was significant ($P<.05$, Student *t* test, $n=5$). The results for the effect of different oxygen concentrations in the target gas for labeling are summarized in Table 2. No significant difference was found in oxygen saturation of HbV at different oxygen concentrations.

In order to further increase the efficiency of radio labeling, the effect of L-cysteine was estimated. Fig. 2 shows a graph of radioactivity yields in $^{15}O_2$-HbV with different concentrations of L-cysteine. Radioactivity of $^{15}O_2$-HbV was significantly higher in HbV samples containing L-cysteine at concentrations of 2.8, 3.0 and 4.0 mM compared with 2.0 mM ($P<.05$), but no significant difference was observed in the radioactivity yield between 2.8, 3.0 and 4.0 mM (ANOVA with post hoc Scheffe's test). From these results, the optimum conditions for $^{15}O_2$-HbV preparation were determined to be 5 ml of HbV containing 2.8 mM L-cysteine and a bubbling time of 2 min with vortexing. Using this method, an average $^{15}O_2$-HbV activity of 214.4±7.8 MBq/mL was achieved ($n=5$).

Table 2
Oxygen saturation at different oxygen concentrations ($n=3$)

| Oxygen concentration (%) | $O_2$ saturation in HbV (%) |
|---|---|
| 2.5 | 77.7±1.7 |
| 5 | 78.0±2.3 |
| 7.5 | 77.8±2.0 |

Values are mean±S.D. The $O_2$ saturation was not significantly different among different conditions ($P=.99$, ANOVA).

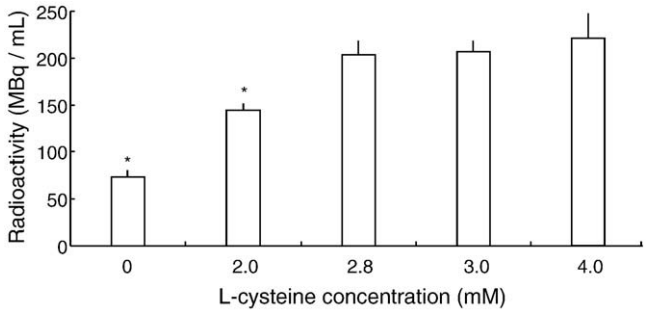


Fig. 2. Radioactivity of $^{15}O_2$-HbV after L-cysteine was added in HbV samples at different concentrations of 0, 2.0, 2.8, 3.0 and 4.0 mM. Values are mean±S.D. ($n=5$ for each condition). *$P<.001$ different from all other conditions by one-way ANOVA with post hoc Scheffe's test.

Endotoxin contamination of HbV samples before and after labeling experiments was found to be below 0.2 EU/mL, which was within permissible limits.

### 3.2. Animal PET studies

Fig. 3 shows the TACs of arterial blood and brain tissue after injection of around 37 MBq of $^{15}O_2$-HbV. Radioactivity in the arterial blood peaked at about 70 s after injection, and brain tissue radioactivity reached a plateau after a gradual increase. Table 3 shows blood gas parameters observed during $H_2^{15}O$ and $^{15}O_2$-HbV PET scans. Arterial blood gas data were not significantly different in the two conditions. Moreover, administration of small volume (1 ml) of $^{15}O_2$-HbV is unlikely to alter the hematological and rheological properties of blood. The mean $tO_2c$ value in five rats studied was 0.22±0.01 during $^{15}O_2$-HbV scans. The mean values of CBF and $CMRO_2$ obtained from five rats

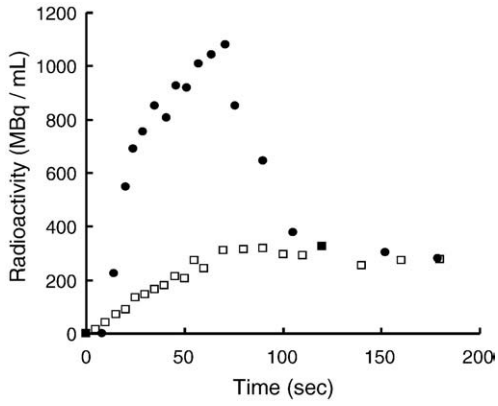


Fig. 3. TACs of arterial blood (●) and brain tissue (□) after the injection of $^{15}O$-$O_2$-labeled HbV ($n=5$) via femoral vein. Rats were anesthetized with choral hydrate (400 mg/kg, ip). The injected dose and volume of labeled HbV were around 37 MBq and 1 mL, respectively.

Table 3
Arterial blood gas data before $H_2O^{15}$ and $^{15}O_2$-HbV PET scans ($n$=5)

| | After $H_2O^{15}$ | After $^{15}O_2$-HbV | Difference |
|---|---|---|---|
| pH | 7.3±0.1 | 7.2±0.1 | n.s. |
| $PCO_2$ (mm Hg) | 47.1±7.7 | 41.1±5.4 | n.s. |
| $PO_2$ (mm Hg) | 77.1±12.5 | 79.5±20.1 | n.s. |
| Hct (%) | 0.6±0.1 | 0.51±0.1 | n.s. |
| $O_2$Sat (%) | 93.5±2.8 | 92.9±4.4 | n.s. |
| Hb (g/dl) | 20.1±1.5 | 20.0±0.6 | n.s. |

Values are means±S.D. n.s., not significant (paired $t$ test).

using $H_2^{15}O$ and $^{15}O_2$-HbV PET were 51.4±7.9 (mL/min per 100 g) and 6.8±1.4 (mL/min per 100 g), respectively. Mean OEF values obtained in this study were 0.61±0.16. Fig. 4 shows typical PET images acquired for 3 min after the injection of 37 MBq of $H_2^{15}O$ and $^{15}O_2$-HbV. The images clearly show the presence of radioactivity in the brain region which demonstrates that $^{15}O$-$O_2$ is carried by HbV in vivo.

## 4. Discussion

Since we wanted to develop a simple system for injectable $^{15}$HbV preparation, a direct bubbling method was selected for labeling. To increase the radioactivity yield in the labeled HbV, various modifications were made to the direct bubbling method to achieve the optimal labeling efficiency. The binding efficiency of $^{15}O_2$-HbV was increased when simultaneous bubbling and vortexing were performed in HbV samples containing L-cysteine. Effects of different concentrations of L-cysteine in HbV on labeling efficacy were compared and the radioactivity of $^{15}O_2$-HbV was maximal at 2.8 mM and higher. The greater labeling efficacy by adding L-cysteine could be attributed to its ability to suppress methemoglobin formation, which is incapable of binding with oxygen, as reported previously [23]. A physiologic sigmoid curve of the hemoglobin–oxygen reaction indicates that the absorption fraction of oxygen in RBCs is greater at a higher partial pressure of oxygen [24]. However, contrary to our expectations, the binding efficiency of HbV did not increase even after changing the partial pressure of oxygen in the target gas. This result can be attributed to the carrier effect [1]. The hemoglobin molecules in HbV are considered to be saturated even at a low concentration of oxygen, and it seems an unimportant factor in deciding the labeling efficiency of HbV. Pressure in the vial was also monitored throughout the bubbling process to achieve the range of 120 to 130 kPa at the maximum, which is close to atmospheric pressure. Although pressure does not seem to affect the hemoglobin binding with oxygen at these values [25], monitoring the experimental conditions during bubbling was helpful to check possible leakage in the system.

HbV is useful to prepare injectable $^{15}O$-$O_2$ because of its smaller size and stability with less wear and tear in vivo, which seemed appropriate for direct bubbling [9,26]. It was shown that a fraction of HbV can bind with $^{15}O_2$ molecules in vivo after inhalation of $^{15}O_2$ gas when it is injected in an

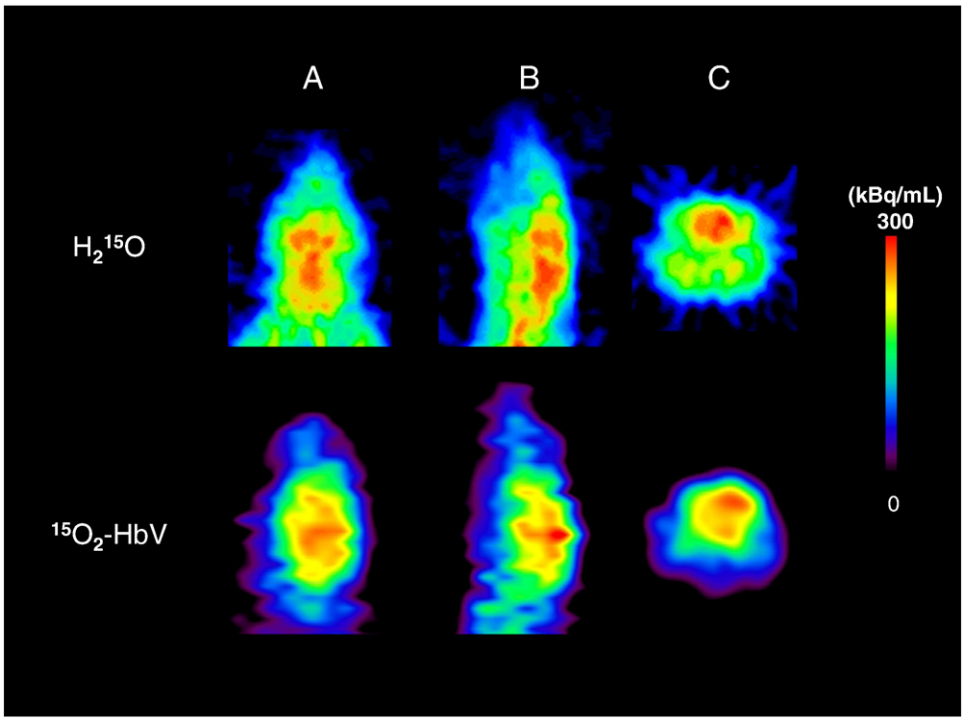


Fig. 4. Small animal PET images of a rat obtained for 3 min after the injection of 37 MBq of $H_2^{15}O$ and $^{15}O_2$-HbV. Coronal (A), sagittal (B) and axial (C) views of a rat brain PET images showing substantial $^{15}O_2$ uptake in the brain.

animal [11]. Moreover, to avoid foam generation when using normal RBCs, the plasma protein fraction has to be separated from whole blood by repeated centrifuging and washing of the RBC fraction, although repeated washing of the RBC fraction may affect their biologic function [5]. Magata et al. [1] reported an injectable $^{15}O$-$O_2$ system using an artificial lung and rat blood for labeling. This method requires a specific system called CUPID to concentrate $^{15}O$-$O_2$ in the target gas because high-radioactivity gas was administered into the artificial lung along with the previously collected rat blood. Therefore, this method requires another animal to be sacrificed for blood collection, and blood clots have to be continuously removed from the collected blood before administration. The target gas and blood have to be circulated at a very high flow rate in the artificial lung to achieve good labeling efficiency. Our HbV-based injectable $^{15}O$-$O_2$ system does not require expensive equipment such as CUPID and an artificial lung, and has no need for prior blood collection from another rat. It requires shorter time to label HbV (2 min) and less radioactivity exposure since the system is semiautomatic and well shielded. Radioactivity thus obtained using the final labeling conditions was greater than 200 MBq/mL HbV on average, which is sufficient activity for animal experiments. Ter-Pogossian et al. [4] reported a yield of 37 MBq/mL of blood when direct bubbling of $^{15}O$-$O_2$ gas was done for 6 min in whole blood though radioactivity of $^{15}O$-$O_2$ gas used for labeling blood was not mentioned. Awasthi et al. [13] reported that radioactivity in HbV by the direct labeling trial with $^{15}O_2$ was only 14.8 MBq/mL of HbV, and thus, the present study is the first report where sufficient radioactivity in the form of injectable $^{15}O_2$-HbV is achieved for carrying out brain oxygen metabolism studies in rats.

CBF values obtained from five rats using the autoradiographic method were consistent with previous reports [1,27], indicating the state of cerebral circulation was in the normal range under anesthesia. CBF values obtained in this study are also not very different from the previous report [2] where same type of anesthesia was used. Since the radioactivity used in measurement of CBF and $CMRO_2$ was 37 MBq because of limitations due to count rate performance of the PET scanner and slow injection rate, it was prudent to use 3-min scan duration for better image quality and contrast. For calculation of CBF, 2–3 min scan duration is used in animal studies and the values obtained are identical [1]. In the $^{15}O_2$-HbV experiments, the tracer was administered with a slow bolus injection and $CMRO_2$ was calculated using two-compartment analysis [22]. $CMRO_2$ values in rats obtained from this $^{15}O_2$-HbV injection method were slightly higher compared with previous report using the same anesthesia [2], although difference in anesthesia may provide a larger difference in $CMRO_2$ as observed in a previous study [1]. They used Mintun's method, which calculates $CMRO_2$ by the combination of CBF, CBV and $O_2$ images [28]. We used Ohta's method which is reported to show a tendency to overestimate oxygen consumption, probably due to the effect of radioactivity in the veins and ignoring recirculation of $^{15}O$-water [29]. However, this simple method with a single injection of $^{15}O_2$-HbV is useful for measuring $CMRO_2$ in small animals because it requires shorter time and the procedure is easier to carry out. Regions of interest were placed on the whole brain region in this study to obtain global values. Local $CMRO_2$ values could be evaluated if PET images are coregistered with MRI images to detect a precise location of changes in regional $CMRO_2$. Moreover, it is also expected that local $CMRO_2$ values can be estimated with a higher-resolution animal PET system.

HbV, a cellular type of hemoglobin-based oxygen carriers (HBOCs), is still in preclinical trial stage and has not yet approved by FDA for human use. Noncellular types of HBOCs are known to produce vasoconstriction due to unbound hemoglobin, but there have been no reports involving HbV which suggests any kind of undesirable effects. Therefore, we believe it can be of potential use in humans though we have no experimental evidence as yet to substantiate our point.

## 5. Conclusion

Our results show that intravenously injectable $^{15}O_2$-HbV could be prepared to carry out quantitative animal studies that successfully calculated $CMRO_2$ in rats. A simple direct bubbling method combined with vortexing and addition of L-cysteine was effective to improve radioactivity yield in $^{15}O_2$-HbV. This simpler and safer injectable $^{15}O$-$O_2$ method will enhance basic researches in various fields and has potential for human use.

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