Research Paper

HFC-152a (1,1-Difluoroethane) inhalation causes a decrease in blood hemoglobin function

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Abstract 1,1-Difluoroethane (HFC-152a) is widely used as a gas component of electronic and precision instrument propellant cleaners. Although HFC-152a has low toxicity, sudden deaths from the abusive inhalation of HFC-152a-containing products have been observed globally. Two cases of HFC-152-related deaths have been reported as the forensic autopsy case. Moreover, we investigated the biodistribution of HFC-152a in rats to elucidate the mechanism of action of HFC-152a. Wistar Rats (n=10) were exposed to the mixed gas (80% HFC-152a and 20% oxygen). They survived the 5-minute exposure but died from asphyxiation after the 10-minute exposure. No significant differences were observed in the biodistribution of HFC-152a between these two groups. Total hemoglobin (tHb) and oxyhemoglobin saturation (O_2Hb) were measured to investigate the influence of HFC-152a on hemoglobin function. HFC-152a did not affect tHb, whereas O_2Hb was significantly decreased by exposure to HFC-152a. Carbon monoxide (CO) is known as a typical toxic gas, and toxicity of CO is known to reduce the oxygen-carrying capacity of blood by forming carboxyhemoglobin. Because similar phenomenon like CO occurred when exposed to HFC-152a, it was suggested that the cause of sudden deaths due to HFC-152a inhalation was asphyxia by decreased O_2Hb level in blood.

Key words: 1,1-difluoroethane, gas inhalation, GC-MS, oxyhemoglobin saturation, distribution of HFC-152a

Introduction

Inhalant abuse is a prevalent form of substance abuse in adolescents and young adults. The most common inhalant substances are aerosol sprays and dusters have been used to induce euphoria by inhaling their volatile components[1−7]. After the 1990s, Japan's most abused volatile substances were butane and propane canisters after the stricter regulations for organic solvents such as toluene[8,9]. A hallmark of their involvement is evidenced by secondary damage, such as explosions. However, deaths associated with inhalant abuse are sparsely reported in Japanese medical institutions.

Hydrofluorocarbons (HFCs) have been used as propellants for commercial and industrial aerosols. The organofluoride compound, 1,1-difluoroethane (HFC-152a) is colorless, odorless and has a lower impact on ozone-depletion compared to other chlorofluorocarbons and hydrochlorofluorocarbons (HFC-134a or HFCF-141b)[10]. Moreover, HFC-152a is used as gas component of spray cleaner of electronic equipment and precision instrument. These HFCs,

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including HFC-152a, are also used apart from normal use among people to induce euphoria by inhaling the gas components[11–14].

Sudden sniffing death syndrome death associated with inhalant abuse[15–22] has occasionally been reported despite the low toxicity of HFC-152a[23]. There is no data on the acute toxicity of HFC-152a, any reports on the blood concentration of HFC-152a postmortem, and whether it was at an intoxication level. We also report two fatal cases involving the inhalation of HFC-152a in our forensic laboratory.

Carbon monoxide (CO) is a known toxic gas, and the toxicity of CO is known to decrease the oxygen-carrying capacity of blood by forming carboxyhemoglobin (CO-Hb)[24]. Post-mortem autopsy findings of CO poisonings are usually classified by asphyxiation and a pink hue on the surface of the decedent's body. It is currently unknown whether HFCs display a similar mechanism of action, which may lead to reduced oxygen-carrying capacity in humans after inhalant exposure. We hypothesize that the oxygen-binding may be affected by HFC-152a exposure in a similar manner.

This investigation aims to report the two fatal cases involving HFC-152a inhalation in our laboratory and to investigate the distribution of HFC-152a via animal experiments to elucidate their mechanism of action on hemoglobin.

Materials and Methods

Chemicals and reagents

A standard of ＞99% HFC-152a was purchased from SynQuest Labs, Inc. (Alachua, FL, USA). For the inhalant exposure study, the air duster canister, Dust Blower (aerosol volume: 460 mL, constituent: ＞99% HFC-152a), was obtained from ELECOM, Co. (Osaka, Japan). In addition, oxygen (＞95%) as diluted gas of air duster was purchased from GL Sciences, Inc. (Tokyo, Japan). The internal standard (IS), 2,2,2-trifluoroethanol, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other solvents and chemicals used were of analytical grade commercially available.

Animal experiment (inhalant exposure study)

All animal experiments were conducted following the *Guidelines for Animal Experimentation* of Osaka University Graduate School of Medicine. Adult male Wistar rats (weighing 250 g) were purchased from CLEA, Japan. Rats were exposed to HFC-152a gas in the exposure chamber (500 cm^3). To avoid the possibility of suffocation due to hypoxia, the mixed gas consists of 80% of HFC-152a and 20% of oxygen. This mixed gas was introduced to the exposure chamber via a flow meter at a flow rate of 3 L/min. The opposite side of the exposure chamber had some holes to allow mixed gas to flow in one direction. After exposure, rats were euthanized by cervical dislocation, whole blood and tissue samples were collected. The collected samples were stored at −80°C until analyzed.

Determination of 1,1-difluoroethane (HFC-152a) in biological samples

Determination of HFC-152a was conducted using a GC-MS headspace method. The working stock solution of HFC-152a was prepared by the water displacement method. The standard HFC-152a gas was transferred into a sealed and weighed glass vial containing 5 mL of distilled water. A final concentration of 0.3 mg/mL HFC-152a was prepared. This 0.3 mg/mL working stock solution was used to create an eight-point calibration curve from 10 to 500 μg/mL. This was accomplished by adding varying amounts of the working stock solution to a sealed 20 mL headspace vials containing 0.8 mL of water, 0.2 mL of negative-control whole blood or 0.2 g of negative-control tissue and 0.1 mL of 2,2,2-trifluoroethanol (IS, 10 mg/mL aqueous solution). In addition, 2 g of the glass bead (average diameter, about 2 mm) was added to the headspace vial to homogenize tissue completely. Gas-tight syringes were used to extract an exact volume of HFC-152a from the vial for calibration. Samples (0.2 mL blood and 0.2 g of each tissue) obtained from the decedents or the sacrificed rats were also added to a sealed 20 mL headspace vials containing 1 mL of water, 0.1 mL of IS aqueous solution and 2 g of the glass bead. Each vial was mixed for 1 min, heated at 70°C for 20 min, and then 100 μL of the headspace was injected into the GC-MS system.

GC-MS system was a Shimadzu GCMS-QP2010 gas chromatography-mass spectrometry (GC-MS) equipped with a CP-PoraBOND Q fused silica column (Agilent Technologies, CA, USA, 25 m×0.25 mm, i.d., thickness 3.0 μm). The injector and detector temperatures were set at 150°C and 200°C, respectively. The column temperature was initially held at 40°C for 2 min, increased to 250°C at a rate of 20°C/min, and held at 250°C for 1 min. A selected ion monitoring (SIM) of GC-MS was used for the quantitative determination of HFC-152a. These ions used for SIM were *m/z* 51 for a fragment ion of HFC-152a (molecular weight

66) and *m/z* 61 for a fragment ion of IS (molecular weight 100).

Determination of total hemoglobin (tHb) and oxyhemoglobin (O_2Hb) in blood

To determine total hemoglobin (tHb) and oxyhemoglobin (O_2Hb) in blood samples after exposure to HFC-152a gas, AVOXimeter 4000 was used (Instrumentation Laboratory, MA, USA). Freshly drawn or anticoagulated whole blood samples were measured according to the manufacturer's protocols. To investigate the effect of HFC-152a on hemoglobin function *in vivo*, rats were exposed to HFC-152a gas according to the abovementioned methods. The whole blood samples collected immediately after exposure was measured.

For an *in vitro* study, healthy adult venous blood was used to investigate the effect of HFC-152a on human hemoglobin function. This venous blood was anticoagulated by adding ethylenediaminetetraacetic acid dipotassium (Merck, Germany). This blood containing the mixed gas (80% of HFC-152a and 20% oxygen) was tightly sealed and shaken gently at 37°C. After a certain period of time, the incubated blood was used for measurement.

Statistical analysis

A one-way analysis of variance followed by the Tukey−Kramer method was used to compare the significance of differences among all groups. All experimental data were expressed as means±standard deviation (SD). Results were significant at $^{*}p<0.05$ and $^{**}p<0.01$.

Case History

Case 1

A 39-year-old male was found on his bathroom floor by his housemate. An empty can of Dust Blower (ELECOM Co., Japan, volume: 460 mL, constituent: HFC-152a) was next to his face. Although bradypnea was reported when he was found, his breathing eventually stopped. He was transferred to the hospital but was dead on arrival. The housemate had recalled seeing his roommate abuse inhalants for several years. An autopsy was performed approximately 18 h after death. Autopsy findings showed acute death from asphyxiation with lung congestion, lung edema, and an enlarged spleen. Some biological samples were collected during autopsy for toxicological analysis and stored at −80°C until analysis.

Case 2

A 27-year-old male was found dead on his bed by his friend. Extensive rigor mortis was reported and the body was transferred to the medical examiner's office for autopsy. The decedent held an air duster, Dust Blower (ELECOM Co., Japan), under his arm, four cans were present around his bed and hundreds of empty cans were found on the porch. An autopsy was performed about 35 h after the discovery of the body. Findings at autopsy revealed significant cyanosis of the lips and face and lung congestions. Several biological samples were collected during autopsy for toxicological analysis and stored at −80°C until analysis.

Results

GC-MS analysis of HFC-152a in biological samples

A quantitative determination for HFC-152a was undertaken using the SIM method of GC-MS analysis. The mass spectra of standard HFC-152a and IS, and a typical SIM chromatogram of HFC-152a in a blood sample (case 1) were shown in Fig. 1. The peaks of HFC-152a and IS had retention times of 3.6 min and 7.2 min, respectively. Moreover, there were no peaks that interfered with the measurements.

The linearity of the calibration curve using the spiked blood and each tissue was observed from 0 to 500 μg/mL (R^2=0.999). The lower limit of quantitation (LLOQ) was about 0.1 μg/mL or μg/g. In addition, the accuracy of HFC-152a measurement in the spiked samples was 93.8−98.7% (data not shown). The coefficient of variation (CV) for HFC-152a measurement was lower than 5.7% at all QC levels (data not shown). Using this method, HFC-152a levels in biological samples obtained at autopsy were determined. When the HFC concentration in the sample exceeded the calibration curve, the amount of the sample used for analysis was appropriately changed and measured.

HFC-152a levels in biological samples obtained from two cases of sudden death after inhaling HFC-152a showed significant variability (Table 1). Blood levels of case 1 and case 2 were 295.1±2.8 and 509.6±2.2 μg/mL, respectively. Urine levels of case 1 and case 2 were 114.8±0.4 and 228.9 ±2.1 μg/mL, respectively. Next, the levels of HFC-152a in case 1 ranged from 39.5±0.3 μg/g in the liver to 138.5± 0.5 μg/g in the lungs. In case 2, ranged from 195.1±1.3 μg/g HFC-152a in the brain to 397.2±2.8 μg/g in the lungs. Other volatile compounds were not detected in all samples.

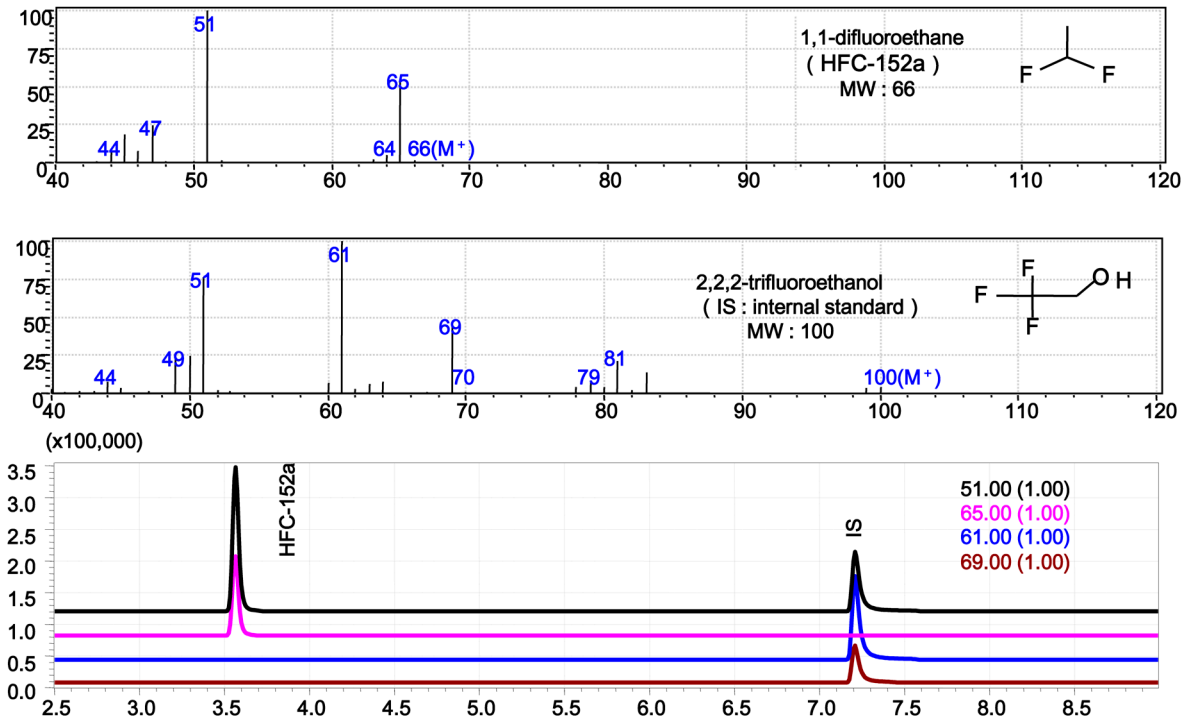

Fig. 1. EI mass spectra of HFC-152a and I.S. (2,2,2-trifluoroethanol) and typical SIM chromatogram of HFC-152a in blood obtained from autopsy sample (case 1).

Table 1. HFC-152a levels in body fluids and various tissues obtained from postmortem samples

Biological samples	Case 1	Case 2
Heart blood	295.1±2.8 μg/mL	509.6±2.2 μg/mL
Urine	114.8±0.4 μg/mL	228.9±2.1 μg/mL
Gastric content	220.0±1.4 μg/mL	382.0±4.8 μg/mL
Brain	85.1±0.3 μg/g	195.1±1.3 μg/g
Heart	42.2±0.3 μg/g	224.3±1.8 μg/g
Lung	138.5±0.5 μg/g	397.2±2.8 μg/g
Liver	39.5±0.3 μg/g	234.7±1.3 μg/g
Kidney	108.7±0.8 μg/g	230.8±1.4 μg/g

The data showed the means ± SD of three determinations.

Distribution of HFC-152a in rats after inhalant exposure

To investigate the biodistribution of HFC-152a after inhalant exposure, rats were exposed to mixed gas according to the methods described above. They were alive during the first 5 min of exposure but died at 10 min of exposure. We compared the distribution of HFC-152a levels in these two groups (survival and death).

In the survival group, the average concentrations of HFC-152a were 377±2 μg/mL (blood), 215±5 μg/g (brain), 166±2 μg/g (heart), 111±5 μg/g (lung), 146±12 μg/g (liver), 178±1 μg/g (spleen), 257±3 μg/g (kidney), and 309 ±2 μg/g (adipose tissue). In the death group, the average concentrations of HFC-152a were 361±14 μg/mL (blood), 239±1 μg/g (brain), 175±9 μg/g (heart), 104±1 μg/g (lung), 122±3 μg/g (liver), 168±7 μg/g (spleen), 234±9 μg/g (kidney), 297±1 μg/g (adipose tissue). There were no significant differences in HFC-152a levels between two groups. For this reason, it was considered that rats in the survival group were placed under a normal typical breathing environment immediately after exposure to HFC-152a, and their activity state returned to the original state with progress.

Change with time of distribution of HFC-152a levels in rats

To investigate the change in time distribution of HFC-152a levels after inhalation exposure, rats were exposed to the mixed gas for 5 min. After certain periods of time (0, 10 and 30 min), rats were euthanized by cervical dislocation and biological samples were collected (survival group). In addition, we also investigated the postmortem change of HFC-152a levels. In survival group, their activities were low for 10 min, whereas these activities recovered to normal about 30 min later.

HFC-152a concentrations in collected biological samples were shown in Fig. 2. HFC-152a concentrations in blood at 10 and 30 min after exposure decreased significantly in the

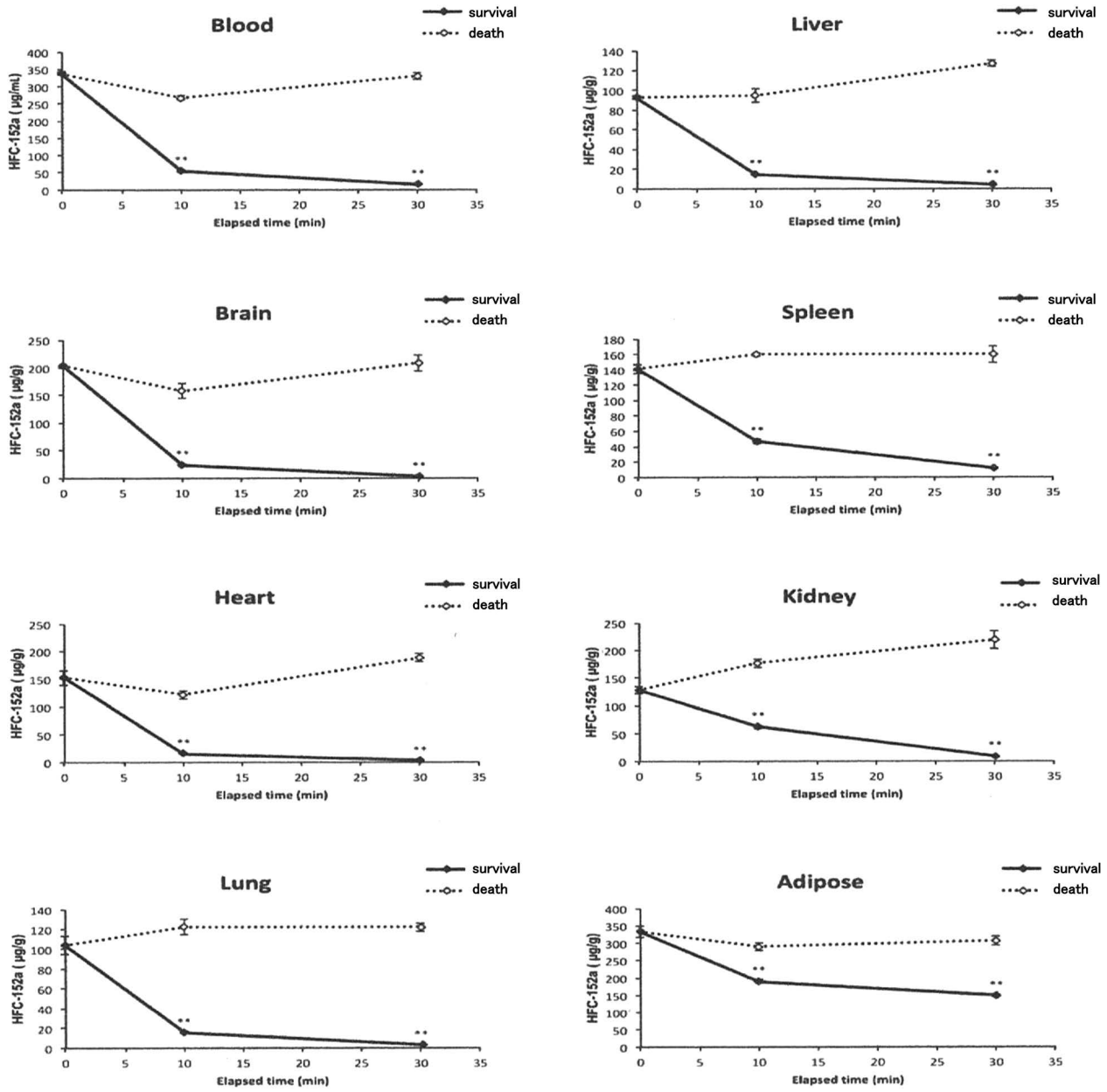

Fig. 2. Changes with time of HFC-152a distribution after exposure.

Rats were exposed to mixed gas (80% HFC-152a and 20% oxygen). The survival means rats that placed under normal breeding environment just after exposure. The death means that rats are euthanized just after exposure and placed under normal breeding environments. After certain period time, biological samples were collected. GC/MS methods quantitated the HFC-152a levels in collected samples. All results were shown as mean±SE (n=10). The statistical significances were shown as ** ($p<0.01$) compared with HFC-152a levels at same sampling time.

survival group, but not in the death group. HFC-152a levels in blood samples obtained from the survival group at 0, 10 and 30 min were 338.1±9.9 μg/mL, 54.5±16.5 μg/mL, and 15.0±6.9 μg/mL, respectively. On the other hand, in the death group, HFC-152a levels at 0, 10 and 30 min were 338.1±9.9 μg/mL, 266.9±19.0 μg/mL and 329.9±38.8 μg/mL, respectively.

HFC-152a concentrations in various tissues obtained from the survival group decreased significantly after 30 min of exposure, while the concentration of HFC-152a remained constant after post-mortem exposure. HFC-152a concentrations in various tissues obtained from the survival group at 0, 10 and 30 min ranged from 93.1±6.8 μg/g, 14.3 ±0.3 μg/g and 3.9±1.1 μg/g in the liver, respectively, to 333.2±44.6 μg/g, 189.8±15.3 μg/g and 148.6±8.3 μg/g in adipose, respectively. In contrast, in death group, HFC-152a levels at 0, 10 and 30 min ranged from 93.1±6.8, 94.8±23.6 and 127.1±10.7 μg/g in the liver, respectively, to 333.2±

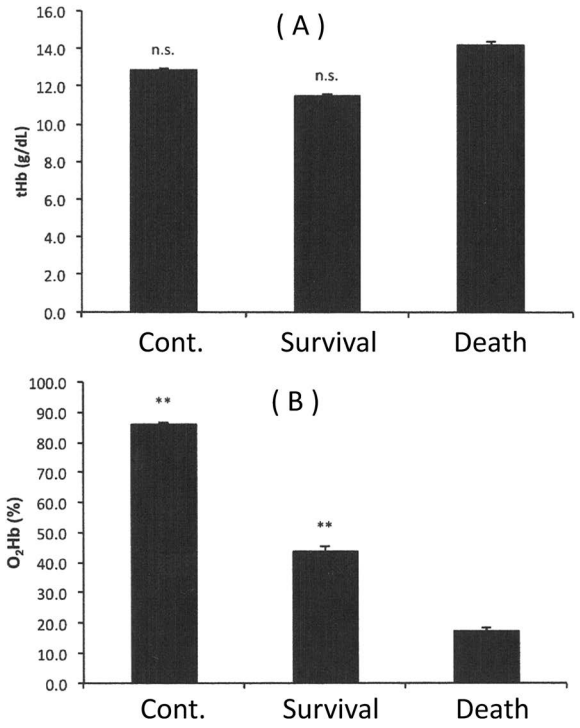

Fig. 3. Influence of HFC-152a on tHb and O_2Hb levels in rat blood after exposure.

(A and B) Rats were exposed to mixed gas (80% of HFC-152a and 20% of oxygen) for 5 min (survival) or 10 min (death). In addition, rats were exposed to artificial air (80% of nitrogen and 20% of oxygen) for 10 min (cont.). After exposure, whole blood was collected and total hemoglobin (tHb) and oxyhemoglobin saturation (O_2Hb) were measured by AVOXimeter. All results were shown as mean±SE (n=10). The statistical significances were shown as follows: n.s.; not significant, **; $p<0.01$ compared with levels of dead.

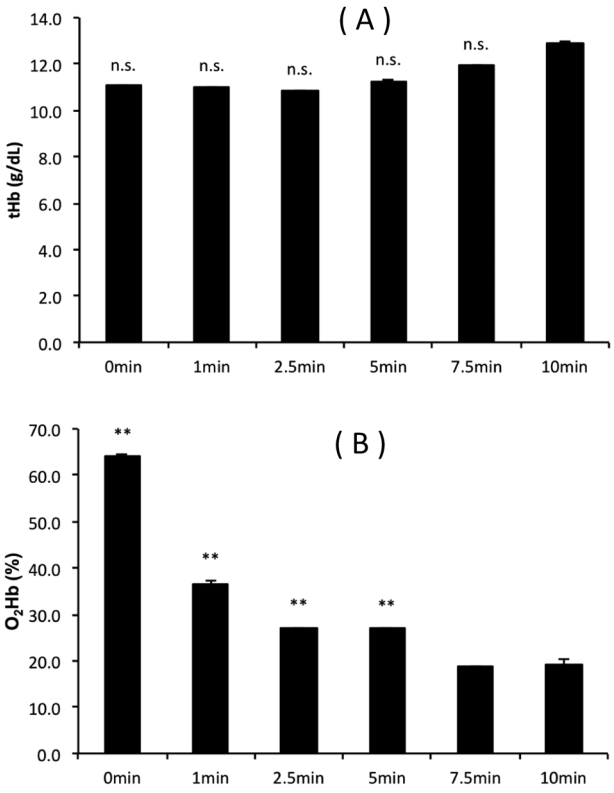

Fig. 4. Influence of HFC-152a on tHb and O_2Hb levels in human blood.

(A and B) The anticoagulated human whole blood was incubated with mixed gas (80% of HFC-152a and 20% of oxygen) for certain period of time. The tHb and O_2Hb of incubated blood were measured by AVOXimeter. All results were shown as mean±SE (n=10). The statistical significances were as follows: n.s.; not significant, **; $p<0.01$ compared with levels at 10 min.

44.6, 289.3±35.8 and 307.1±49.3 μg/g in adipose, respectively.

Influence of HFC-152a to hemoglobin function

The total hemoglobin (tHb) and oxyhemoglobin saturation (O_2Hb) in collected samples were measured by AVOXimeter according to the methods mentioned above. Then, to investigate the influence of HFC-152a to hemoglobin function, rats were exposed to the mixed gas for 5 min (survival group) and 10 min (death group). Rats exposed to artificial air (80% of nitrogen and 20% of oxygen) for 10 min were applied as a control group. In both groups, whole blood was collected from the heart after being euthanized. In the death group, whole blood was collected after exposure to HFC-152a. In Fig. 3(A) and 3(B), levels of tHb and O_2Hb in blood collected after exposure to HFC-152a were shown, respectively. No significant difference in tHb score was recognized among these three groups. However, the O_2Hb score significantly decreased in the death group compared with other groups (survival and control).

Next, we investigated whether a similar influence occurred on human hemoglobin *in vitro*. The anticoagulated human whole blood was mixed with the gas were shaken gently at 37°C for a certain period. The incubated blood samples were used for the measurement of tHb and O_2Hb. The tHb and O_2Hb were shown in Fig. 4(A) and 4(B), respectively. The tHb score did not recognize remarkable change derived from the difference in incubation time. However, O_2Hb score significantly decreased depending on incubation time.

Discussion

Our autopsy cases observed remarkable differences in HFC-152a levels in postmortem samples. As the cause of these differences between the two cases, we assumed that the death process differed. The victim in case 1 was alive when he was found, whereas the victim in case 2 was

already dead when he was found. Generally, the blood concentration of toxicants has been used as a diagnostic index of poisoning. In our autopsy cases, HFC-152a levels in the heart blood of case 1 and case 2 were 295.1 μg/mL and 509.6 μg/mL, respectively. These blood levels were higher than previously reported autopsy cases[1–4,16,17]. Moreover, the cause of death obtained from these autopsy cases has been estimated to be asphyxiation or fatal cardiac arrhythmia[2,25,26]. However, the actual mechanism of death due to inhalation of HFC-152a is still unknown.

Therefore, the biodistribution of HFC-152a was preliminarily investigated by animal experiments. First, we examined rats' HFC-152a levels in the blood after inhalant exposure, but no statistical difference in HFC-152a levels in blood was found between survival and death groups. These results suggest that it is difficult to use the blood level of HFC-152a as an index of HFC-125a poisoning and that a new index is needed.

Next, the changes in HFC-152a distribution after inhalation exposure were investigated. Rats survived the 5 min exposure to HFC-152a, and their locomotion recovered over time. In these survival groups, the immediate decrease of HFC-152a levels was shown in all collected samples except for adipose. However, no significant changes were observed in all samples collected in the death group, which was euthanized after 5 min exposure to HFC-152a. In addition, there was a tendency for HFC-152a levels to increase slightly even 30 min after exposure in the liver, brain, heart, and kidney. Regarding this point, the possibility of redistribution of HFCs among tissues is considered, but it is not clear. These findings suggested that HFC-152a was rapidly eliminated via respiration. Therefore, it was thought that HFC-152a levels in postmortem samples were difficult to use as an index of HFC-152a poisoning. Furthermore, the rapid elimination of HFC-152a by respiration may account for the differences in HFC-152a levels observed between our autopsy cases.

The distribution of inhaled HFC-152a has been reported in rats[27] and humans[28]. It has been reported that repeated exposure of HFC-152a to rats tended to accumulate in cardiac tissue. In addition, according to inhalation experiments of HFC-152a in humans, HFC-152a tends to be stored in adipose tissue. Our inhalation experiments in rats also showed no significant reduction in adipose tissue, although other tissues showed rapid reduction after 30 min. Based on the previous reports, including ours, it seems that no toxicological features have been found in terms of the biodistribution of HFCs.

Carbon monoxide (CO) is a known toxic gas. In Japan, many poisoning cases due to CO inhalation occur every year[29]. The toxicity of CO was known to decrease the oxygen-carrying capacity of blood by forming abnormal CO-Hb[24], eventually leading various poisoning symptoms by reduced oxygen partial pressure. Therefore, we investigated whether HFC-152a has similar effects to CO. Rats were exposed to HFC-152a for 5 min (survival group) or 10 min (death group). For control group, rats were exposed to artificial air for 10 min. As the result, no significant difference in tHb score was recognized among these 3 groups. However, O_2Hb score significantly decreased in the death group compared with other groups (survival and control).

Moreover, human whole blood samples incubated with HFC-152a did not show any significant change in tHb. In contrast, the O_2Hb score significantly decreased depending on incubation time. From these results, it was revealed that HFC-152a was able to reduce O_2Hb *in vivo* and *in vitro*. Therefore, the likely mechanism of action of HFC-152a was thought to be asphyxia by decreasing the O_2Hb level. HFC-152a likely has a high binding affinity to hemoglobin as the number of O_2Hb decreases, which contributes to asphyxia. In addition, the possibility of abnormal HFC-Hb formation by HFC-152a inhalation was beyond the scope of this study that should be investigated in future reports.

According to the previous reports[1–4,15–22], blood levels of HFC-152a in fatal cases vary from 83.5 to 546 μg/mL. The present blood levels in two cases were within those in previously reported fatal cases and then it was considered that HFC-152a levels in blood were lethal. The differences in HFC-152a levels were observed between both cases. HFC-152a levels of case 1, which was transferred to the emergency unit immediately after discovery, were significantly lower than case 2, which was found dead on. It has been reported that exhalation[9,18] rapidly eliminates HFC-152a. Therefore, it is considered that lower HFC-152a levels of case 1 result from the elimination by exhalation.

Conclusion

Using an animal exposure model, we demonstrated that asphyxia due to decreased O_2Hb was likely the toxic mechanism of HFC-152a inhalation. Therefore, the evaluation of HFC-152a poisoning should be carried out by O_2Hb score, and it is difficult to evaluate the HFC-152a poisoning by

HFC-152a levels in the blood or other tissue sample. In addition, the risk of HFC-152a inhalation has been underreported, and aerosol products should be regulated to avoid similar poisoning cases.

Ethical Standards

This article complies with the current law of Japan.

Conflict of Interest

The authors report no conflict of interest.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with international and national committee ethical standards and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Moreover, this study was undertaken in accordance with the ethical standards set by Osaka University School of Medicine's institutional review bord. Informed consent was obtained from all participants included in the study. The analysis of toxic substances from authentic blood samples was permitted by judicial authorities and supported by official documentation.

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