J Occup Health 2015; 57: 161–168

Journal of Occupational Health

Possible production of arsenic hemoglobin adducts via exposure to arsine

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Abstract: Possible production of arsenic hemoglobin adducts via exposure to arsine: Takenori YAMAUCHI, *et al*. Department of Public Health Faculty of Medicine, University of Miyazaki—Objectives: Arsine is an arsenic compound generated as a by-product in metal refineries. Accidental poisoning occurs sporadically; however, the administrative level for workers has not been established. Thus, it is essential to identify a highly specific biomarker for risk management in the workplace. The aim of this study was to identify an arsenic adduct, a potential biomarker, in the plasma. **Methods:** Preserved mouse blood was exposed to arsine *in vitro*, and the plasma was separated. The residual clot of the control sample was hemolyzed using ultrapure water, and the supernatant was collected. Plasma from mice exposed to arsine *in vivo* was also separated from blood. Immunoprecipitation assays were conducted using all samples after ultrafiltration, and three fractions were collected. The total arsenic concentration in each fraction was quantified using inductively coupled plasma mass spectrometry (ICP-MS). The three *in vitro* samples and the eluate fraction from immunoprecipitation were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). **Results:** In the exposed samples, the arsenic concentration in the fraction containing immunocomplexes was higher when immunoprecipitation was conducted with an anti-globin antibody. Three peaks were specifically observed in arsine-exposed samples after MALDI-TOF-MS analysis. Two of them were around *m/z* 15,000, and the other was *m/z* 15,700. The latter peak was confirmed even after immunoprecipitation. **Conclusions:** Globin forms an adduct with arsenic after both *in vitro* and *in vivo* exposure to arsine. This adduct together with hemoglobinuria could be a candidate biomarker of acute arsine poisoning in plasma.
(J Occup Health 2015; 57: 161–168)

Key words: Acute poisoning, Adduct, Arsine, Biomarker, Hemoglobin

Arsine (AsH_3) is a gaseous arsenic compound that has been designated as a toxic substance by the Poisonous and Deleterious Substances Control Act in Japan. It is synthesized by certain microorganisms (e.g., *Pseudomonas* and *Alcaligenes*) under anaerobic conditions; and as a by-product of metal acid treatments and refining processes when arsenic impurities are present[1–3]. Thus, accidental arsine poisoning can occur in the refining industry[4–6].

Acute symptoms of arsine poisoning include cephalgia, malaise; and vomiting, followed by hemolysis, hematuria; and pyrexia in later stages. In severe cases, anemia and renal failure can lead to death[7, 8]. In particular, acute arsenic poisoning is characterized by hemolysis observed several hours after exposure. However, although an occupational exposure limit for arsine has been established, the control concentration has not. As a result, it is likely that many work floors do not have sufficient environmental controls to limit exposure. Thus, the development of a biomarker for risk management in the workplace is essential. Although hemoglobinuria is considered a biomarker of arsine exposure, its specificity is not

Received Jun 26, 2014; Accepted Dec 2, 2014
Published online in J-STAGE Jan 20, 2015
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high; because it is observed following acute intoxication with other heavy metals such as copper and lead. Thus, a highly specific biomarker of arsine intoxication is also required. Recent reports have described an unidentified arsenic compound derived from blood cells with a molecular weight over 10,000 after *in vitro* exposure of blood to arsine[9]. Furthermore, it was indicated that the arsenic coexisted with hemoglobin in red blood cells separated from mice exposed to arsine[10]. The aim of this study was to identify an arsenic adduct that can serve as a candidate biomarker in both plasma separated from preserved mouse blood exposed to arsine *in vitro* and plasma from mice exposed to arsine *in vivo.*

Materials and Methods

Sample preparation

Preserved whole blood derived from male ICR mice was purchased from Nippon Bio-Supp. Center and was exposed to arsine *in vitro*. Arsine was generated according to Japanese Industrial Standard (JIS) K 0102; by reducing 2 mg of arsenic trioxide with hydrochloric acid and zinc powder. The preserved blood was exposed to the generated arsine for 10 minutes as described previously[9]. Whole blood was diluted with an equal volume of Alsever's anticoagulant solution (2.05 g glucose, 0.42 g sodium chloride, 0.80 g sodium citrate, and 0.055 g citric acid per 100 m*l*). The plasma derived from the blood exposed to arsine *in vitro* was called "Exp_ivt", and that derived from control blood was called "Cont_ivt". An equal volume of ultrapure water was added to the residual clot from the control blood and was mixed by vortexing. The suspensions were centrifuged to remove cell debris. The supernatant was collected; and was used for analysis; it was referred to as "Cont_Lys_ivt".

Blood samples exposed to arsine *in vivo* were obtained in a previous study[10]. Briefly, male Hos: HR-1 hairless mice were placed in an acrylic exposure chamber and exposed to 2.5 *l* of arsine gas for 5 minutes under isoflurane anesthesia. Animal experiment protocols were approved by the Ethical Committee of Nihon University (approval #: AP11P042). All experiments with animals were conducted in compliance with the Ethical Guidelines for Animal Experiments of Nihon University. The exposure experiment was conducted in a fume hood for safety. Arsine was generated by reduction of arsenic trioxide with alkaline $NaBH_4$. The arsine concentration was 320 ppm at 25°C, as measured by a Kitagawa-type detector tube system. All mice were euthanized with isoflurane, and the blood from each mouse was collected transcardially using a heparin-treated syringe. Blood plasma was separated from the whole blood by centrifugation; and was called "Cont_ivv". Similarly, the plasma from the blood derived from arsine-treated mice was separated by centrifugation and called "Exp_ivv".

Ultrafiltration of samples

Ultrafiltration of all samples was conducted with a MINICENT-30 (Tosoh Bioscience Japan, Tokyo, Japan) and Vivaspin 500; 3 kDa MWCO (GE Healthcare Japan, Tokyo, Japan). First, 50 μl of each sample was mixed with 150 μl of Dulbecco's phosphate-buffered saline (-) (DPBS(-)). To remove debris, the samples were centrifuged at 3,000 rpm for 10 minutes at 4°C, followed by centrifugation at 15,000 rpm for 10 minutes. The supernatant was applied to the MINICENT-30 (molecular weight cut-off: 30 kDa), and samples were centrifuged at 3,000 g for 100 minutes. The upper fraction, containing proteins greater than 30 kDa, was recovered in 200 μl of DPBS (-) and called the "high" fraction. The lower fraction was used for ultrafiltration. The fraction was applied to the Vivaspin 500; 3 kDa MWCO (molecular weight cut-off: 3 kDa) and centrifuged at 15,000 rpm for 100 minutes. The upper fraction, with a molecular weight over 3 kDa and below 30 kDa, was also recovered in 200 μl of DPBS (-) and called the "mid" fraction. The "mid" and "high" fractions of each sample were used for subsequent experiments.

Immunoprecipitation

The two fractions ("mid" and "high") of the three *in vitro* samples (Exp_ivt, Cont_ivt and Cont_Lys_ivt) and two *in vivo* samples (Exp_ivv and Cont_ivv) were used for the immunoprecipitation assay (IP). Three groups were created per sample; depending on the antibody. For Group 1, 60 μl of each sample was mixed with 440 μl of DPBS(-). In group 2, 60 μl samples were mixed with 0.5 μl of anti-α-hemoglobin antibody (ab92492, Abcam, Cambridge, MA, USA), 2.5 μl of anti-β-hemoglobin antibody (sc-31116, Santa Cruz Biotechnology, Santa Cruz, CA, USA); and 437 μl of DPBS(-). Finally, for group 3, 60 μl of each sample was mixed with 0.5 μl of anti-β-actin antibody (GTX109639, Gene Tex, Irvine, CA, USA) and 439.5 μl of DPBS(-). The total volume of each sample was adjusted to 500 μl. The samples were rotated overnight at 4°C to allow antigen-antibody interaction. Protein G Sepharose 4 Fast Flow (10 μl, GE Healthcare) was added to each sample to absorb the antigen-antibody complexes, and the samples were rotated overnight at 4°C. The samples were centrifuged at 3,000 rpm for 10 minutes at 4°C to separate the supernatant and the precipitates. The former fraction was defined as the "Sup" fraction. The latter

fraction was washed ten times with DPBS(-). Finally, 50 μl of elution buffer (Protein A/G HP SpinTrap™ Buffer Kit, GE Healthcare) was added to each sample, followed by centrifugation at 3,000 rpm for 10 minutes at 4°C to remove the antigen-antibody complex from the sepharose beads. This fraction of the supernatant was defined as the "Eluate", and the precipitate was defined as the "Beads".

Inductively-coupled plasma mass spectrometry (ICP-MS)

The total arsenic concentration contained in each sample was quantified by ICP-MS (iCAP Qc ICP-MS, Thermo Fisher Scientific Inc., Waltham, MA, USA). Ultrapure water for analysis was prepared using a Milli-Q Element A-10 with a Quantum ICP cartridge (Merck Millipore Japan, Tokyo, Japan). Each sample was diluted with 1% nitric acid (Ultrapure-100 nitric acid 1.42, Kanto Chemical Co., Inc., Tokyo, Japan). The "Sup" fraction (100 μl) was diluted with 4,900 μl of 1% nitric acid. Further, 10 μl of the "Beads" fraction was diluted with 590 μl of 1% nitric acid, followed by centrifugation at 3,000 rpm for 10 minutes at 4°C to remove sepharose beads. Finally, 30 μl of sample from the "Eluate" fraction was diluted with 570 μl of 1% nitric acid. The instrument settings were as follows: RF power, 1,550 W; plasma argon gas flow rate, 14 *l*/min; carrier argon gas flow rate, 1.0 *l*/min; auxiliary gas flow rate 0.8 *l*/min; helium gas flow rate, 4.5 *l*/min; electric voltage for kinetic energy discrimination, +3V. A concentric nebulizer, nickel sampling cone; and nickel skimmer cone were used. The ICP-MS detection mass was set as *m/z* 75 ($^{75}As^{+}$). The limit of detection (LOD) was defined as the concentration corresponding to three times the standard deviation of the blank ion intensity. Similarly, the limit of quantification (LOQ) was 3.3 times the LOD. The LOD and LOQ were 0.007 ppb and 0.023 ppb, respectively. The calibration curve was estimated using six point standards (0.0125 ppb to 10 ppb, R=0.99997) with an arsenic standard solution (Arsenic ICP-MS Standard, AccuStandard, Inc., New Haven, CT, USA). Arsenic concentrations below the LOQ were defined as not detectable (ND) because it was essential for our study to compare the arsenic concentrations among samples.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Three plasma samples (Exp_ivt, Cont_ivt, and Cont_Lys_ivt) were analyzed by MALDI-TOF-MS (Autoflex III, Bruker Daltonics, Yokohama, Japan). The "Eluate" fraction obtained by IP of the "high" fraction of Exp_ivt was also analyzed by MALDI-TOF-MS (Autoflex II, Bruker Daltonics). Each sample was diluted 75-fold with a 0.1% solution of trifluoroacetic acid. In preparing the matrix solution, 20 mg of sinapinic acid was dissolved in 50 μl of TA solution (one volume of acetonitrile and two volumes of 0.1% solution of trifluoroacetic acid) according to the manufacturer's protocol. One volume of the diluted sample was mixed with two volumes of matrix, and 1 μl of the mixed solution was spotted on the target plate. The spectra were acquired by irradiating the plasma sample and IP sample with a laser, with integration of a positive linear mode in one spectrum. The range of detection was from *m/z* 2,000 to 90,000. Double-digit *m/z* acquisitions by MALDI-TOF-MS analysis were ignored; because an error of 500 ppm from the true value was accepted during calibration.

Results

Detection of arsenic-bound globin by immunoprecipitation

The total arsenic concentration in the "mid" fraction of the "Sup", "Beads" and "Eluate" fractions from *in vitro* samples was quantified by ICP-MS. All samples with the exception of the "Sup" fraction of Exp_ivt; were below the LOQ. The arsenic concentrations were 0.78 ± 0.03, 0.78 ± 0.03 and 0.77 ± 0.02 ppb when IP was conducted with no antibody, anti-α- and β-hemoglobin antibodies and β-actin antibody, respectively. The arsenic content of the "high" fraction of the *in vitro* sample was also quantified (Table 1). The arsenic concentrations in the "Beads" and "Eluate" fractions of Exp_ivt were much higher when the IP was conducted using anti-α- and β-hemoglobin antibodies. Similarly, the arsenic concentration in the "mid" and "high" fraction of the *in vivo* samples was quantified after IP. These results did not differ much from those of the *in vitro* samples. In the "mid" fraction, only the arsenic concentration of the "Sup" fraction of Exp_ivv was above the LOQ. The arsenic concentrations were 0.03 ± 0.004, 0.03 ± 0.007 and 0.04 ± 0.006 when IP was conducted with no antibody, anti-α- and β-hemoglobin antibodies and β-actin antibody, respectively. Further, the concentration in the "Eluate" fraction of Exp_ivt was higher only when the IP was performed using anti-α- and β-hemoglobin antibodies (Table 2).

Detection of specific peaks in samples exposed to arsine in vitro

Mass spectra of the three *in vitro* samples were obtained between *m/z* 2,000 and 20,000. Four notable peaks were confirmed. Two of them were confirmed at *m/z* 7,500; and 7,800 (data not shown), and the other two peaks were at *m/z* 15,000 and 15,600 (Fig. 1A). In order to magnify the peak at *m/z* 15,000, another experiment was conducted to reveal

Table 1. Total arsenic concentration in the "high" fractions of the *in vitro* samples (ppb)

Sample	Fraction	Antibody		
		(-)	Anti-α- and β-Hemoglobin	Anti-β-Actin
Exp_ivt	Eluate	0.21 ± 0.01	0.73 ± 0.02	0.20 ± 0.01
	Beads	0.71 ± 0.01	1.66 ± 0.02	0.79 ± 0.02
	Sup	2.32 ± 0.04	2.11 ± 0.06	2.31 ± 0.05
Cont_ivt	Eluate	ND	ND	ND
	Beads	ND	ND	ND
	Sup	ND	ND	ND
Cont_Lys_ivt	Eluate	ND	ND	ND
	Beads	ND	ND	ND
	Sup	ND	ND	ND

The arsenic concentration determined by ICP-MS is presented as the mean ± standard deviation (SD) of five measurements. ND means the concentration of arsenic was below the limit of quantification (LOQ).

Table 2. Total arsenic concentration in the "high" fractions of the *in vivo* samples (ppb)

Sample	Fraction	Antibody		
		(-)	Anti-α- and β-Hemoglobin	Anti-β-Actin
Exp_ivt	Eluate	0.26 ± 0.01	0.32 ± 0.01	0.26 ± 0.01
	Beads	0.14 ± 0.02	0.14 ± 0.01	0.14 ± 0.01
	Sup	0.52 ± 0.01	0.50 ± 0.02	0.48 ± 0.02
Cont_ivt	Eluate	ND	ND	ND
	Beads	ND	ND	ND
	Sup	ND	ND	ND

The arsenic concentration determined by ICP-MS is presented as the mean ± standard deviation (SD) of five measurements. ND means the concentration of arsenic was below the limit of quantification (LOQ).

a trimodal peak in Exp_ivt (Fig. 1B). The first and third peaks were considered to be specific for Exp_ivt; because no equivalent peaks were observed in other samples. In Cont_Lys_ivt, small shoulders could be seen near the first and third peaks of Exp_ivt, but they slightly deviated from these peaks. The peak at *m/z* 15,600 was not specific for Exp_ivt, because it was also observed in the Cont_ivt and Cont_Lys_ivt samples. However, the nearby peak at *m/z* 15,700 was specifically confirmed in Exp_ivt (Fig. 1A), because it was not observed in other samples even when all samples were enlarged around *m/z* 15,700. The peaks at *m/z* 7,500 and 7,800 were considered to be bivalent ions of the peaks at *m/z* 15,000 and 15,600, respectively. Four groups of peaks were detected at *m/z* 32,000, 48,000, 63,000 and 77,000 when the *m/z* range was increased to over 20,000. The peak group at *m/z* 77,000 was observed only in the Exp_ivt and Cont_Lys_ivt samples. In contrast, the other groups were observed in all samples (Fig. 1C). Mass spectra of the "Eluate" fraction obtained by IP of the Exp_ivt "high" fraction using anti-α- and β-hemoglobin antibodies are shown in Fig. 2. Both the peaks at *m/z* 15,000 and 15,700 were confirmed, although the peak at *m/z* 15,600 was not comparable to the peak at *m/z* 15,000.

Discussion

Exposure to arsine through inhalation causes severe hemolysis. However, the administrative level is not established, and few biological exposure indices have been assessed. Thus, it is essential to develop a

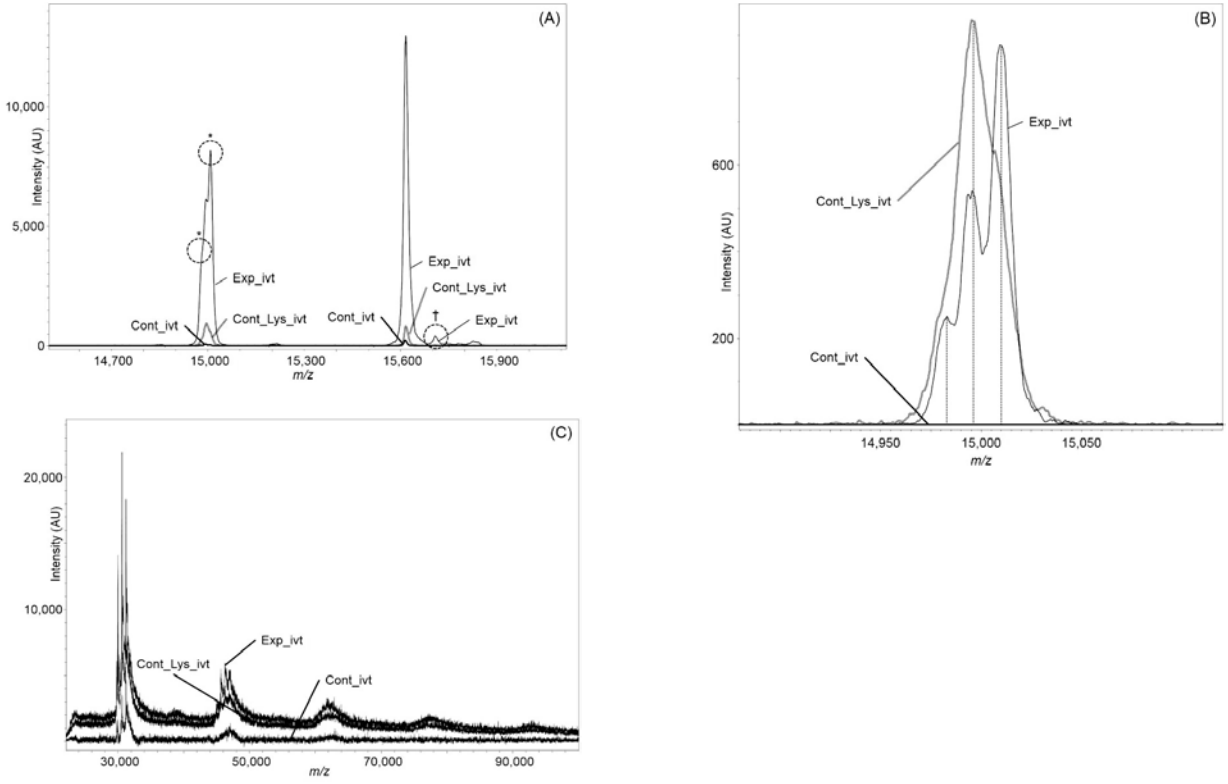

Fig. 1. Three *in vitro* samples (Cont_ivt, Cont_Lys_ivt and Exp_ivt) were analyzed by MALDI-TOF-MS. (A) Peaks at *m/z* 15,000 and 15,600. The * and † symbols show the specific peaks for Exp_ivt at *m/z* 15,000 and 15,700 respectively. (B) The magnified peaks of Cont_ivt, Cont_Lys_ivt and Exp_ivt at *m/z* 15,000 obtained by another experiment in which Exp_ivt was irradiated with much less laser than Cont_ivt and Cont_Lys_ivt in order to additionally examine the peak of Exp_ivt. (C). The peaks at *m/z* over 20,000.

biomarker to manage arsine exposure risk in the workplace. In this study, we detected an arsenic adduct to hemoglobin via IP followed by ICP-MS analysis in plasma separated from both whole blood exposed to arsine *in vitro* and mice exposed to arsine *in vivo*, and a molecule with a molecular weight 15,700 was suspected to be contained in the adduct via MALDI-TOF-MS analysis.

The utility of chemical adducts to hemoglobin as biomarkers of chemical exposure has been reported[11]. These adducts are useful exposure biomarkers because the level of chemical adducts to hemoglobin increases proportionally with the level of toxin exposure, as was previously confirmed in the cases of styrene, styrene oxide, acrylamide and acrylonitrile, which are designated as class I chemical substances by the Japanese Pollutant Release and Transfer Register law[12–23]. Furthermore, 2-chlorovinyldichloroarsine (lewisite), which is an arsenic compound, also forms an adduct to hemoglobin[24]. In this study, we confirmed the binding of arsenic to hemoglobin via IP followed by ICP-MS and indicated the possibility of the arsenic adduct being a candidate biomarker, as is reported in other chemicals.

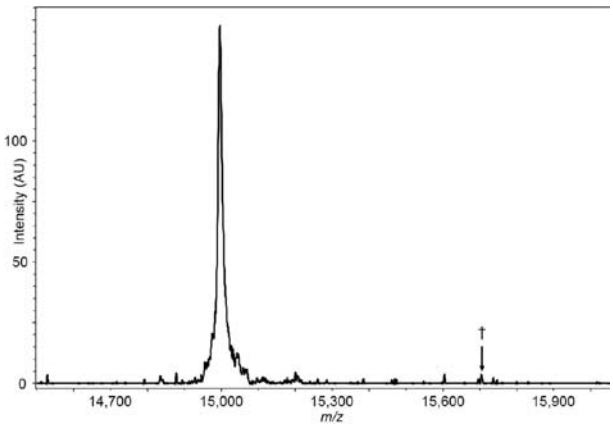

Fig. 2. The peaks in the "high" fraction of the *in vitro* samples obtained by IP at between *m/z* 15,000 and 15,600 observed by MALDI-TOF-MS analysis. The † symbol shows the same peaks as in Fig. 1.

Hemoglobin is a major component of the cytoplasm of red blood cells (RBCs). It is composed of four heme molecules, two α-globin molecules and two

β-globin molecules. Generally, the methionine located on the N-terminus is removed during maturation of the protein. Previous data indicate that the N-terminal methionine of α-globin and β-globin are removed in a similar manner[25]. According to the Swiss-Prot database, the molecular weights of immature mouse α-globin and β-globin are estimated to be 15,085 Da and 15,840 Da, respectively. The molecular weights of matured α-globin and β-globin, in which the N-terminal methionine is removed, are approximately 14,982 and 15,618, respectively, according to ProteinProspector. These results are consistent with a previous study reporting the molecular weights of α-globin and β-globin[26]. Hence, it is possible that one of the trimodal peaks observed at *m/z* 15,000 is derived from α-globin, although the derivation of the other peaks that were specific to "Exp_ivt" remains to be elucidated. The peak at *m/z* 15,600 is derived from β-globin. Furthermore, the peak at *m/z* 15,700 is hypothesized to be "α-globin + heme + arsenic", "β-globin + arsenic" or "α-globin + 8 × arsenic" because the molecular weights of arsenic and heme are 75 and 616, respectively. Arsenic was not detected in the "mid" fraction of the "Eluate" and "Beads" fractions of both Exp_ivt and Exp_ivv. In contrast, it was detected in the "high" fractions of the "Eluate" and "Beads" fraction. Therefore, the arsenic adduct would exist as a form of dimer, trimer or tetramer because the molecular weight of the particles in the "high" fraction was over 30,000 via ultrafiltration. The group of peaks observed at *m/z* 32,000 and 48,000 contains degradation products or bivalent ions of hemoglobin, since the molecular weight of mouse hemoglobin is 63,664. Furthermore, it is also possible that the group of peaks was formed via the omission of heme from hemoglobin; and the degradation of hemoglobin or the ion in it. However, the derivation of the peak observed at *m/z* 77,000 remains to be elucidated.

A recent study indicated that trivalent arsenic adduct formation occurred when hemoglobin or separated RBCs from rats and humans were incubated with inorganic arsenic, monomethylarsonic acid (MMA^{III}) and dimethylarsinic acid (DMA^{III})[27]. Further, the longer retention time for arsenic in rats than in humans; is likely due to the affinity of trivalent arsenic for sulfhydryl groups of the cysteine contained in hemoglobin because of the larger number of the cysteines in rat hemoglobin. A different study reported that trivalent inorganic arsenic has a strong affinity for adjacent cysteine residues; and can form coordinated architecture with cysteine residues[28–30]. Both mouse α- and β-globin contain only one cysteine. Therefore, it is consistent with our result if arsenic binds to cysteine even in the case of arsine exposure. However, arsenic predominantly bound to α-globin when DMA^{V} was orally administered to rats even though the numbers of cysteines contained in α- and β-globin were 3 and 2, respectively. Thus, unknown factors associated with adduct formation were indicated, and further study is necessary to clarify them. Meanwhile, an *in vitro* model was established in a previous study to evaluate hemolysis via arsine exposure[31]. Using that model, different types of hemoglobin were exposed to arsine, and it was found that the type of hemoglobin responsible for hemolysis was primarily oxygenated hemoglobin (HbO_2)[32]. Two possible mechanisms were proposed to explain this effect. One is reaction of arsine directly with HbO_2, causing destabilization of the protein, degradation of hemoglobin and subsequent hemolysis. The other is generation of the arsenic product by the reaction of arsine with HbO_2 but not the reaction of arsine itself with HbO_2 or methemoglobin, inducing hemolysis. Our results indicating the arsenic adduct would possibly support the latter hypothesis. Furthermore, a ternary DMA^{III}-hemoglobin-haptoglobin (As-Hb-Hp) complex was detected in the plasma of rats to which arsenite was orally administered[33]. However, arsenic adduct formation was not observed in plasma when whole human blood or hemolyzed blood was mixed with trivalent inorganic arsenic *in vitro*[9], and there are no reports of hemolysis due to trivalent arsenic exposure. Therefore, it is likely that trivalent arsenic does not induce hemolysis and that the arsenic adduct is not detected in plasma, although the trivalent arsenic has affinity to hemoglobin in RBCs. In line with this, the As-Hb-Hp complex can be released in plasma via physiological degradation of RBCs, and it is possible that the mechanism of generation of the arsenic adduct would differ. It is essential to conduct toxicological studies to determine the mechanism underlying adduct formation in arsenic exposure.

A recent report suggests that arsenic adducts on hemoglobin or globin are not generated when human hemoglobin and RBCs are exposed to arsine *in vitro*[34]. There are three main reasons that likely enabled us to detect the arsenic adducts in our study. First, the exposure concentration of arsine, which was maintained at roughly 80 ppm for at least 30 minutes, was higher than that of previous studies, which used 31 ppm. The second reason we observed adduct formation was that the quantity of the arsenic adduct formed was larger. Previous work using styrene oxide[13] and acrylamide[23] showed that there was a dose-dependent increase in hemoglobin adduct formation. Therefore, it is likely that production of the arsenic adduct would change in a dose-dependent manner after arsine exposure. The higher exposure concentration in our study induced the formation of

more arsenic adducts, allowing us to detect formation. The final reason is the experimental design. In the previous study, human hemoglobin was exposed to arsine, and α- and β-globin were analyzed by Liquid chromatography–mass spectrometry (LC-MS). In contrast, in our study, α- and β-globin in plasma were concentrated by IP, and arsenic was detected by ICP-MS. Together, these reasons explain why we may have observed arsenic adduct formation *in vitro* when previous studies did not.

One limitation of our study is that it remains unclear whether the arsenic adduct is derived from α- or β-globin; due to the mixed use of anti-α-globin and anti-β-globin antibodies in IP. Another limitation was different methods of arsine generation. In the *in vitro* experiment, arsine was generated based on JIS K0102, similar to a previous study; however, the generation of chlorine gas as a by-product was of concern. A second method that did not produce a by-product gas was adopted in the *in vivo* experiment. Therefore, it is possible that chlorine gas could have caused additional hemolysis. Furthermore, it is likely that the hemoglobin peak was observed in the "Cont_ivt" fraction because of hemolysis in the preserved blood due to incomplete handling. Finally, the peak at *m/z* 15,600 was not comparable to the peak at *m/z* 15,000 in the "Eluate" fraction even though IP was conducted with both anti-α- and β-hemoglobin antibodies. The reason remains to be elucidated.

Out study strongly indicated that arsine exposure generates arsenic adducts on globin. Adduct formation in blood plasma could be a candidate biomarker of acute arsine poisoning in conjunction with hemoglobinuria.

Acknowledgments: This study was supported by a Health Labour Sciences Research Grant for Research on Occupational Safety and Health (23250301) from the Ministry of Health, Labour and Welfare of Japan and JSPS KAKENHI Grant Number 25860463.

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