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Evaluation of Chromium in Red Blood Cells as an Indicator of Exposure to Hexavalent Chromium: an *In Vitro* Study.

Jérôme Devoy*[1], Antoine Géhin[2], Samuel Müller[1], Mathieu Melczer[1], Aurélie Remy[1], Guillaume Antoine[1] and Isabelle Sponne[3]

[1]*Laboratory of Toxicology, Institut National de Recherche et de Sécurité, Rue du Morvan, CS 60027, F-54519 Vandoeuvre-les-Nancy, France.*

[2]*ISTerre, University of Grenoble, Maison des Geosciences, 1381 Rue de la Piscine, F-38400 St Martin d'Hères, France.*

[3]*Laboratory of Immunology, Institut National de Recherche et de Sécurité, Rue du Morvan, CS 60027, F-54519 Vandoeuvre-les-Nancy, France.*

*Corresponding author (Jérôme Devoy)

Tel: +33-3 83 50 20 43; Fax: +33-3 83 50 20 96; E-mail address: jerome.devoy@inrs.fr

Abstract

Chromium(VI) compounds are classified as carcinogenic to humans. Whereas chromium measurements in urine and whole blood (i.e., including plasma) are indicative of recent exposure, chromium in red blood cells (RBC) is attributable specifically to Cr(VI) exposure.

Before recommending Cr in RBC as a biological indicator of Cr(VI) exposure, *in-vitro* studies must be undertaken to assess its reliability. The present study examines the relationship between the chromium added to a blood sample and that subsequently found in the RBC.

After incubation of total blood with chromium, RBC were isolated, counted and their viability assessed. Direct analysis of chromium in RBC was conducted using Atomic Absorption Spectrometry.

Hexavalent, but not trivalent Cr, was seen to accumulate in the RBC and we found a strong correlation between the Cr(VI) concentration added to a blood sample and the amount of Cr in RBC. This relationship appears to be independent of the chemical properties of the human blood samples (e.g., different blood donors or different reducing capacities).

Even though *in-vivo* studies are still needed to integrate our understanding of Cr(VI) toxicokinetics, our findings reinforce the idea that a single determination of the chromium concentration in RBC would enable biomonitoring of critical cases of Cr(VI) exposure.

Keywords: Chromium, chromate, dichromate, erythrocyte, RBC, blood, ICP-MS, GF-AAS, biomonitoring.

Abbreviations:

AA: Ascorbic Acid

ACGIH: American Conference of Governmental Industrial Hygienists

BEI: Biological Exposure Indicator

CrA: Chromium in Atmosphere

CRI: Collision Reaction Interface

CrIE: Chromium in Erythrocyte (in RBC)

ECHA: European Chemicals Agency

GEQUAS: The German External Quality Assessment Scheme (For Analyses in Biological Materials)

GF-AAS: Graphite Furnace Atomic Absorption Spectroscopy

ICP-MS: Inductively Coupled Plasma – Mass Spectrometry

PRC: Plasma Reduction Capacity

QMEQAS: Quebec Multielement External Quality Assessment Scheme

RBC: Red Blood Cells or erythrocytes

SVHC: Substance of Very High Concern

TLV-TWA: Threshold Limit Value - Time Weighted Average

1. Introduction

Chromium was first discovered in 1780 by the French chemist, Nicolas Louis Vauquelin, in a mineral sample of 'Siberian red lead'- now known as crocoite (lead chromate). Chromium is a silver, lustrous and very hard metal that can take a high mirror polish, and it is odorless, tasteless and malleable (Weeks, 1932). Chromium compounds are valued as pigments due to their vivid green, yellow, red and orange colors. The metal is also widely used for its catalytic properties. Chromium is used in stainless steel, contributing to resistance to oxidation, and when combined with nickel or vanadium (and tungsten), contributes to ductility and resistance to temperature, respectively. Chromium can exist in oxidation states ranging from -2 to +6. Of these, pure metal (Cr(0)), trivalent (Cr(III)) and hexavalent chromium (Cr(VI)) can all be encountered in the working environment. Metallic chromium dust may pose a health risk in the workplace but the risk can easily be reduced through the use of respiratory protection and/or technical devices. Cr(III) is an essential dietary nutrient for humans and is considered nontoxic (Nurminen, 2004). Upon skin contact, both Cr(III) and Cr(VI) may cause irritation and frequently produce allergic reactions (affecting 1 to 3 % of the general population) (Hansen et al., 2003; Thyssen and Menne, 2010; Yoshihisa and Shimizu, 2012). This contact dermatitis could easily be prevented by wearing protective clothing and gloves to avoid dermal exposure. Among the forms of chromium encountered in workspace, only Cr(VI) compounds are recognized as carcinogenic and are classified as SVHC by the ECHA (IARC, 1990; INRS, 2006). Monitoring of this species is therefore of particular importance.

Industrial operations such as refractory steel processing, stainless steel production, welding, chrome plating, tanning (Hedberg et al., 2015) and chromite ore-processing may expose employees to these elements. In metrological approaches, monitoring of exposure to these metals combines analysis of the air with analysis of biological environments such as urine or blood. In Europe, the recommended indicative limiting values for professional exposure are atmospheric chromium (CrA) concentrations of 2 mg/m³ (8-hour average) for Cr(0), for the inorganic chromium compounds (Cr(II)) and for the (insoluble) inorganic chromium compounds (Cr(III)) (European Commission, 2006). In France, professional exposure limit values (VLEP) also exist for the compounds of Cr(VI) (0.001 mg Cr/m³) and Cr trioxydes (0.05 mg Cr/m³) (JO, 2012). In the United States, the ACGIH (American Conference of Governmental Industrial Hygienists) recommends the use of limiting values based on actual occupational exposure: the TLV (Threshold Limit Value). The TLV-TWA (Threshold Limit Value - Time Weighted Average) exposure limit value corresponds to a concentration accumulated over either an 8-hour workday or a 40-hour working week. The ACGIH

recommends a value of 0.5 mg Cr/m³ for chromium metal and for the compounds of Cr(III), a value of 0.05 mg Cr/m³ for Cr(VI) and a value of 0.01 mg Cr/m³ for insoluble Cr(VI) (ACGIH, 2013).

While analysis of CrA is well-documented from a methodological point of view, and is therefore used for regulatory purposes, there is a lack of data concerning the analysis of chromium in biological samples, even though chromium in these samples reflects the real exposure experienced by a worker.

In recent decades, a number of surveillance techniques have been developed that allow internal exposure to Cr(VI) to be monitored through analysis of biological fluids such as urine, blood and plasma. A number of investigations have helped to establish the relationship between airborne chromium and levels of chromium in blood, plasma and urine (Alexopoulos et al., 2008; Gube et al., 2010; Matczak et al., 1195; Miksche and Lewalter, 1997; Mutti et al., 1979; Mutti et al., 1984; Stridsklev et al., 2004; Tola et al., 1977). Urinary chromium can be regarded as a reliable marker of internal chromium exposure and is sufficiently sensitive for biological monitoring of exposure levels below the occupational limits. However, on the basis of urinary chromium alone, it is not possible to distinguish between exposure to Cr(VI) and exposure to Cr(III). Only chromium in erythrocytes is diagnostic of internal exposure to Cr(VI). This is because hexavalent chromium, unlike Cr(III) complexes, is able to cross the cell membranes of red blood cells (RBC) via anion carrier proteins (Kortenkamp et al., 1987). Chromium in lymphocytes has also been suggested to be a good indicator of Cr(VI) exposure (Lukanova et al., 1996).

Before validating the use of chromium in RBC and/or lymphocytes as an eventual Cr(VI) exposure biomarker, in vitro verification experiments should be performed. The main goal of this study is to check for linearity between the amounts of Cr(VI) added to blood samples and the concentrations of Cr measured in erythrocytes. We tested several parameters including the chromium oxidation state (oxidation states 3 and 6), chromium counterion (K^+, Na^+ and NH_4^+), chromium concentration (1 µg/L to 1 g/L), incubation temperature (4 to 37°C), incubation time (up to 24 hours), added ascorbic acid (AA) concentration (0 to 100 mg/L) and different blood donors. To study the influence of these parameters, whole blood samples were spiked with various quantities of highly-water-soluble Cr(VI) and Cr(III) compounds and the chromium levels in the RBC and lymphocytes were then determined. Only data for the RBC are presented in this paper.

2. Experimental Methods

2.1. Apparatus

A Varian AA280Z atomic absorption spectrophotometer, equipped with a Zeeman background corrector, was used for atomic absorption measurement of chromium at 357.9 nm and with a slit-width of 0.5 mm. A hollow cathode Cr lamp (SCP Science, 030-150-244) was operated at 3 mA. Uncoated graphite tube cuvettes were purchased from Schunk Kohlenstofftechnik (Germany).

The Vi-CELL Cell Viability Analyze system, composed of the analyzer instrument and software (Beckman Coulter, Miami, USA), was used to determine cell concentration and viability. This system provides an automated means of performing the trypan blue dye exclusion method. The Vi-CELL instrument was calibrated using the Beckman Coulter Vi-CELL concentration control, following standard Beckman Coulter procedures. Each measurement consisted of the acquisition of fifty individual images per sample. Vi-CELL reagent packs were used as instructed in the Beckman Coulter, Inc instruction manual.

2.2. Reagents and solutions

All chemicals used in the study were of analytical grade or higher. Nitric acid was used to prepare 0.2% HNO_3 (v/v) with ultrapure water. All single element stock solutions (1000 mg/L) were delivered by SCP Science and were certified for purity and concentration. From these stock solutions, a mixed working-standard solution with a concentration of 10 mg/L for each element was prepared by adding 1 mL of each stock solution to a 100-mL measuring flask, then adding 5 mL of purified HNO_3 and diluting to 100 mL with ultrapure water (MilliQ, Millipore, Germany).

Chromium standard solutions for calibrations were prepared by diluting a 10 g/L chromium standard stock solution (140.002.24x, SCP Science) with 2% v/v HNO_3.

$Na_2Cr_2O_7$ (Alfa Aesar, 43180, 99.999%), $(NH_4)_2Cr_2O_7$, $K_2Cr_2O_7$ (Sigma-Aldrich, 216615, 99%), Na_2CrO_4 and K_2CrO_4 were tested for Cr(VI), and $CrCl_3$ (Sigma Aldrich, 27096) was selected for Cr(III). Ascorbic acid (analR NORMAPUR, 20150) was used to estimate blood reducing capacity.

Quality control materials (such as standard or certified reference materials) are routinely used in analytical laboratories to ensure the accuracy of both the instrument and the analytical procedure. In this work, six blood metal control materials (QMEQAS blood control (QM-B-Q1403, QM-B-Q1404, QM-B-Q1409 and QM-B-Q1410) and GEQUAS (49/2012 1A, 49/2012 1B)) and two plasma metal control materials (GEQUAS (49/2012 11A, 49/2012 11B)) were used.

External quality assurance was carried out through participation in the following international comparison programs and quality assessment schemes:

- the German External Quality Assessment Scheme (G-EQUAS) at the Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine at Friedrich-Alexander-University Erlangen-Nuremberg, Germany;
- the Quebec Multielement External Quality Assessment Scheme (QMEQAS) at the Canadian Institut National de Santé Publique du Québec.

2.3. Human blood samples

A sufficient number of 500-mL human blood bag units were purchased from the local hospital to meet our research needs. The blood donors were all anonymous. Anticoagulant Citrate Phosphate Dextrose solution is systematically present in the blood bag units. Citrate was chosen due to its negligible influence on the accumulation of Cr(VI) in RBC (Afolaranmi et al., 2010).

2.4. Collection and preparation of biological samples

In order to eliminate any possible Cr contamination, all plastic ware was soaked in 20 % HNO_3 for 24 hours prior to use and rinsed three times with deionized distilled water. Each 500 mL human blood bag unit was drawn into fifty 10-mL top metal-free Vacutainer tubes. Each Cr concentration (represented by individual points in the figures) was duplicated at least once. Hematocrit 1 (Ht 1) was determined in the 500 mL human blood bag units using a hematocrit centrifuge.

Chromium was added to 10-mL Vacutainer tubes of blood.

Samples were left for 40 minutes at room temperature to allow the blood to separate into two fractions: Fraction 1, the supernatant, which contained the plasma and the white blood cells; and Fraction 2, which contained predominantly RBC. The second fraction was diluted with 3 mL of NaCl solution (0.9 %) up to the initial volume of 15 mL, then divided into three aliquots, left to stand at room temperature for 10 min, and finally centrifuged for 10 min at 3000 rpm. The washing procedure was then repeated twice more using 0.9 % NaCl solution. The pellets were re-suspended very gently to prevent disruption of the erythrocyte membrane. Hematocrit 2 (Ht 2) was determined just before the third washing step. The erythrocyte pellets (RBC) were diluted to the initial volume of the blood sample and Triton X-100 was added at 0.1 %. Cell counting and viability determinations were performed by V-Cell counter.

2.5. Direct analysis of Cr using GF-AAS

Direct analysis of chromium in whole blood and RBC cells was conducted using a Varian AA280Z. To reduce sample viscosity and dispense the sample more evenly in the graphite tube, aliquots of the blood samples were further diluted by a factor of 1:5 by adding 2% v/v HNO_3 and 1.0% v/v Triton X-100. RBC samples were also diluted by a factor of 1:5. 30 µL of diluted sample was introduced to a graphite tube and atomized according to a set temperature program (Table 1). Peak areas in absorbance were used for quantitative analysis. Chromium calibration solutions were prepared in 0.2% v/v HNO_3 and 1.0% v/v Triton X-100. Method quantification limits for the original samples (before dilution or treatment) were 0.4 µg/L in RBC and total blood.

2.6. Validation of analytical methods

In order to validate our analytical method, we followed the same procedure as that described in an earlier paper in which we assessed analysis of beryllium in human urine samples (Devoy et al., 2013). The validation procedure provides estimates of the linearity limit, precision (intermediate precision, repeatability and reproducibility), accuracy and sensitivity, for analysis of the element in question. The procedure used is also similar to that proposed by Peters et al. (Peters et al., 2007).

2.7. Statistical analysis

The RBC chromium data were analyzed using a linear mixed-effect model after log-transformation of the data. Chromium in RBC was expressed either as a fraction (%) or as mass per number of cells (µg/10^6 RBC). The fixed effects were the chromium species, the chromium concentration on the logarithm scale and the counterion. The random effect was the "Donor", to take into account the variability between the different donors. The statistical significance level was set at 5%. All statistical analyses were performed with Stata 13.0.

3. Results and discussion

3.1.Cr(VI)/Cr(III) in RBC

To validate the use of the chromium concentration in erythrocytes as an eventual biomarker of Cr(VI) exposure, only Cr(VI), and therefore no Cr(III), should penetrate the membrane of the erythrocytes.

Chromium concentrations in RBC are well-established for monitoring of critical cases of Cr(VI) exposure (Lewalter et al., 1985). Radiolabeled sodium chromate (^{51}Cr(VI)) is commonly used

for diagnostic purposes for *in vitro* and *ex vivo* RBC marking (Coogan et al., 1991). Dianionic chromate ions appear to bind to the red blood cell in two steps: first by a rapid but reversible attachment to the cell membrane and then by a slower near-irreversible binding to intracellular hemoglobin and reduction to the anionic state (Qu et al., 2008). It has been suggested that the slow rate of uptake is dependent on the rate at which chromate can penetrate the cell membrane. Binding is maintained until the red blood cells have been sequestered by the spleen or until the chromium is eluted into the plasma. The chromium is then readily excreted, mainly in the urine.

Cr(III) ions cross the membranes slowly by simple diffusion (Cohen et al., 1993; Kotenkamp et al., 1987). Cr(III) RBC membrane penetration is not well understood but plays an important role that should be taken into account when considering RBC chromium as a specific Cr(VI) exposure biomarker.

In order to check the selectivity of the RBC membrane regarding chromium oxidation states, different concentrations of chromium (Cr(VI) or Cr(III)) (2 µg/L to 1 g/L) were spiked in a 10-mL metal-free Vacutainer tube of blood. $(NH_4)_2Cr_2O_7$ and $CrCl_3$ were tested for Cr(VI) and Cr(III), respectively. Two measurements were made for each concentration.

Figure 1A shows the fraction of chromium (Cr(VI) or Cr(III)) that accumulated in the RBC as a function of the amount of chromium in the blood. At concentrations of up to 20 µg/L, 100 % of the Cr(VI) was detected in the erythrocytes. In contrast, at the same concentrations, the fraction of Cr(III) in the RBC reached a maximum of 21 % and was commonly less than 5 %. As the concentration of Cr(VI) in the blood was increased from 30 µg/L to 50 mg/L, the fraction of Cr(VI) in the erythrocytes decreased gradually, to a value of 73 %. A sharp decrease in the fraction of Cr(VI) in the erythrocytes was observed at Cr(VI) concentrations above 50 mg/L, to a value of 15% at a concentration of 1 g/L. During biomonitoring of workers exposed to soluble Cr(VI) during chrome-plating (Goldoni et al., 2010), electrodeposition (Lukanova et al., 1996) or electro-welding (Ivanenko, 2011), maximum Cr concentrations of 20 µg/L in RBC and whole blood have been reported. In this concentration range, our data show that 100 % of the Cr(VI) is located in the RBC. At Cr concentrations above 50 mg/L, the cellular viability was still higher than 95% and no effect on viability was observed for higher chromium concentrations. Consequently, maximum chromium accumulation in the cells appears to have been reached. Chromium is naturally present in RBC and this may explain the fractions of over 100 % recorded for Cr(VI), or fractions above zero for Cr(III).

In Figure 1B, we examine the same data, this time focusing on the range of possible occupational chromium concentrations. The data are expressed as Cr in RBC versus Cr in blood. For Cr concentrations of 3 to 100 µg/L, the Cr in RBC for Cr(VI) is proportional to the Cr added to the

blood ($y = 0.0271x + 932.16$; $R^2 = 0.9$). In contrast, the Cr in RBC for Cr(III) stays below 0.6 µg/10^6 cells, and is comparable to the blank concentration (0.24 ± 0.23 µg/10^6 cells).

For biomonitoring purposes, and because a large decrease in the fraction was observed at concentrations above 100 mg/L, we performed statistical analysis only on the data from added chromium concentrations of up to 100 mg/L. After log-transformation of the data, the slopes of the mass of chromium in the RBC as a function of the mass of chromium added to the blood sample can be calculated. The slopes determined for Cr(III) and Cr(VI) were 0.48 [0.42-0.53] and 0.99 [0.96-1.01], respectively, and the difference in slope is significant ($p < 0.001$).

Eight blank tests were performed to estimate the residual chromium concentration in blood and RBC. Concentrations of 2.8 ± 1.6 µg/L and 0.28 ± 0.27 µg/10^6 cells, respectively, were determined/measured. The chromium blood concentration is lower than that reported in Kucera et al. (4.2 ± 1.1 µg/L, n=10) for Czech and Slovak populations (Kucera et al., 1995), and is slightly higher than the concentrations reported in White and Sabbioni (0.19 µg/L, n=134) for a population from the United Kingdom (White and Sabbioni, 1998) and by Christensen et al. (0.18 µg/L, n=23) for a Danish population (Christensen et al., 1993).

3.2.The effect of speciation of hexavalent chromium

Speciation of hexavalent chromium may influence the accumulation of chromium in RBC. Chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) were added at different concentrations (ranging from 2 µg/L to 1 g/L) to 10 mL blood samples. The results are illustrated in Figure 2A.

After log-transformation of the data, the slopes (and 95 % confidence intervals) of the chromate and dichromate data are 0.97 [0.93-1.00] and 0.99 [0.97-1.02], respectively. The difference in slope is not significant ($p > 0.05$).

The influence of the counterion associated with dichromate (K^+, Na^+ and NH_4^+) was also investigated (Fig. 2B). The slopes for potassium, sodium and ammonium are 0.95 [0.90-1.00], 1.00 [0.97-1.03] and 1.04 [0.97-1.11], respectively. The differences in slope are not significant ($p > 0.05$).

The speciation of hexavalent chromium (chromate and dichromate associated with K^+, Na^+ and NH_4^+) therefore appears to have had no effect on its accumulation in RBC.

3.3. The effect of incubation temperature and time.

Incubation parameters such as time and temperature may also have a strong influence on the fraction of chromium in RBC.

Different incubation temperatures were tested for a 40-min incubation time (Fig. 3A): 4, 10, 15, 20, 22, 30 and 37°C. The fraction of chromium detected in the RBC was over 50 %, even at the

lowest temperatures, and the fraction increased over temperatures up to 20°C. The highest fraction (more than 98 %) was reached at temperatures above 20°C.

Different incubation times were then tested at room temperature (22°C) (Fig. 3B): 1, 2, 5, 10, 40, 120, 360, 960, 1440 minutes. After 2 minutes of incubation, 89 % of the hexavalent chromium had accumulated in the RBC, and a plateau was observed at between 10 and 40 minutes. These results are very similar to those obtained with chromate on human leucocytes (Lilien et al., 1970). The appropriate *in-vitro* incubation conditions required to reach a plateau therefore appear to be 40 minutes at room temperature.

In comparison, chromium in RBC derived from trivalent chromium added to blood samples at a concentration of up to 10 mg/L and an incubation time of up to 24 hours, reached no more than 3 $\mu g/10^6$ RBC, i.e., a maximum of 0.2 % of the total chromium.

One limitation of this study is that the effect of longer incubation times could not be assessed due to the difficulty in keeping RBC alive for more than 24 hours. However, we note that Weber (Weber, 1983) reported constant RBC chromium values for up to 15 days following intratracheal chromate exposure in rats.

3.4. Influence of PRC and blood donors

Human plasma has a spontaneous reduction capacity that allows it to detoxicate up to 2 ppm of Cr(VI) compounds into Cr(III) compounds through the formation of excretable Cr-protein complexes (Korallus et al., 1984). The plasma reduction capacity (PRC) can be explained by the combined presence of a multitude of reducing substances. One such substance, glutathione, also has the capacity to reduce Cr(VI) to Cr(III) in RBC via an intracellular mechanism (Wiegand et al., 1984), in effect helping to trap the Cr(VI) in the RBC. For this study, we chose to use another reducing agent, ascorbic acid (AA), to examine the influence of the PRC.

In order to assess the influence of AA on chromium accumulation in RBC, increasing amounts of AA were added to 10-mL blood samples taken from a single donor. Different concentrations of AA were tested (Fig. 4): 0, 1, 2, 3, 5, 7, 10, 20, 50 and 100 mg/L. As the concentration of added AA was increased from 0 to 20 mg/L, the fraction of Cr in the RBC decreased, but still remained at over 87 %. At concentrations above 20 mg/L, the decrease in the fraction continued. The average concentration of AA present in human blood is 8.9±0.3 mg/L (ranging from 1 to 25 mg/L) (Moran et al., 1993). Thus, though AA may have an influence on chromium accumulation in RBC, it is probably not significant in most human blood sample cases. This may suggest that the PRC is not strong enough to prevent hexavalent chromium from penetrating the RBC. This is consistent with the

findings of Qu et al. (Qu et al., 2008) but contrary to those of Harzdorf et al.(Harzdorf and Lewalter, 1997).

As different blood donors can also have different PRC, we examined the dichromate accumulation capacities of blood samples from four different donors (Fig. 5). The data points in Figure 5 all lay on the same trend and, furthermore, all statistical analyses were able to integrate the variability of the donor with the use of linear mixed effects models, including the random effect, "Donor".

3.5. Cytotoxicity of hexavalent chromium

With our incubation parameters (40 minutes at room temperature), and even when using chromate and dichromate concentrations of up to 1 g/L, the viability of the RBC remained at over 95%. The mean viabilities were 96.4 % ± 0.6 % (n=8) for blank samples, 97.8 % ± 0.3 % (n=22) for RBC exposed to Cr(III) and 96.9 % ± 0.8 % (n=123) for RBC exposed to Cr(VI). There are no significant differences between these means. However, while no cytotoxicity was indicated under these conditions, different results might be obtained at another incubation temperature or over a longer incubation time.

3.6. Application to biological monitoring

In this study, chromate and dichromate, but not Cr(III), were seen to accumulate in the RBC. This suggests that RBC selectively accumulate Cr(VI). Because Cr(VI) but not Cr(III) penetrates RBC, and because RBC have a long residence time in the body (up to 120 days), RBC might therefore be a sensitive indicator of Cr(VI) exposure. We also observed a strong correlation between the Cr(VI) concentration added to a blood sample and the amount of Cr measured in RBC. This relationship appears to be independent of certain chemical properties of the human blood samples (e.g., different blood donors or different concentrations of AA commonly found in blood samples).

In an earlier study, Gao et al. monitored the total chromium present in rat fluids following intratracheal administration of soluble trivalent and hexavalent chromium compounds (Gao et al., 1993). Wistar rats exhibited increased chromium concentrations in whole blood, plasma and urine up to 72 h post exposure. The ratio of whole blood chromium to plasma chromium concentrations differed significantly for Cr(VI) and Cr(III) treatments. They therefore recommended that both blood chromium and plasma chromium assays be used for assessment of chromium exposure. They concluded their article by pointing out the potential of blood cells for use as biomarkers for the assessment of exposure to chromium compounds.

Given these findings, we can conclude that Cr in RBC may be a good indicator of hexavalent chromium exposure. Several authors have studied the relationships between chromium levels in lymphocytes, RBC and urine, and ambient air exposure among "chromium workers" and some interesting correlations were found. Nevertheless, Cr-RBC still needs to be validated as a hexavalent chromium exposure indicator. This validation may prove quite difficult however, since RBC and lymphocytes have different lifetimes, of approximately 115 days, and months to years, respectively. The very variable lifetimes of lymphocytes could thus make their use as an exposure indicator somewhat difficult. In contrast, RBC-Cr should be representative of the amount of hexavalent chromium accumulated within the cells over the preceding eight to ten weeks (Miksche et Lewalter, 1997).

Our study has highlighted that hexavalent chromium, unlike trivalent chromium, enters RBC quantitatively, regardless of any differences between the blood samples analyzed (e.g. different blood donors or different AA concentrations). The final step will be to firmly link hexavalent chromium exposure to measured RBC chromium. A significant amount of in vitro and in vivo data should be collected in order to confirm this relationship and to allow RBC-Cr to be proposed as a biological indicator of hexavalent chromium exposure.

4. Conclusions

The core objective of this *in vitro* study was to confirm that the RBC selectively accumulate hexavalent chromium. This would allow Cr-RBC to be proposed as an indicator (or biomarker) of exposure to hexavalent chromium and the data could be used/experiments could be performed to verify the possible toxic effect of chromium species on RBC.

The different forms of soluble chromium were added to blood samples and Cr concentrations were then measured in the RBC and whole blood. A linear relationship, with a slope close to 1, was observed between the concentration of chromium added to the blood and the hexavalent chromium content subsequently measured in the RBC. In contrast, the penetration of trivalent chromium in the RBC was found to be almost nonexistent.

To evaluate the veracity of our results and assess the robustness of Cr-RBC as an indicator, different parameters were tested: hexavalent chromium speciation and the associated counterion, incubation parameters (temperature and time), the reducing capacity of the blood, and the blood donor.

In the context of biomonitoring, concentrations of chromium and ascorbic acid in the blood are unlikely to exceed 100 µg/L and 20 mg/L, respectively. Under these conditions, the results of this study therefore confirm that the Cr-RBC is a good candidate for an indicator of the internal dose resulting from recent exposure (during the preceding 8-10 weeks) to hexavalent chromium. The

proposed method provides rapid and simple determination of ultratrace amounts of chromium in RBC samples and is appropriate for the biomonitoring of occupationally-exposed people in the chromium-using industry. The method allows analysis of chromium in RBC samples at concentrations as low as 0.4 μg/L, a level low enough to meet that required for environmental biomonitoring.

In terms of the appearance of hexavalent chromium toxicity, Cr (VI) accumulates in RBC at levels of up to 1 g / L without any effect on cell viability for a 40 minute incubation time. A bearing effect appears to start at 1 g/L, which might be interpreted as/might reflect the onset of chromium saturation in RBC.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank the National Institute for Research and Safety. We particularly acknowledge the efforts of Alice Williams, Anne-Marie Lambert-Xolin, Elodie Bonfanti and Constance Devoy.

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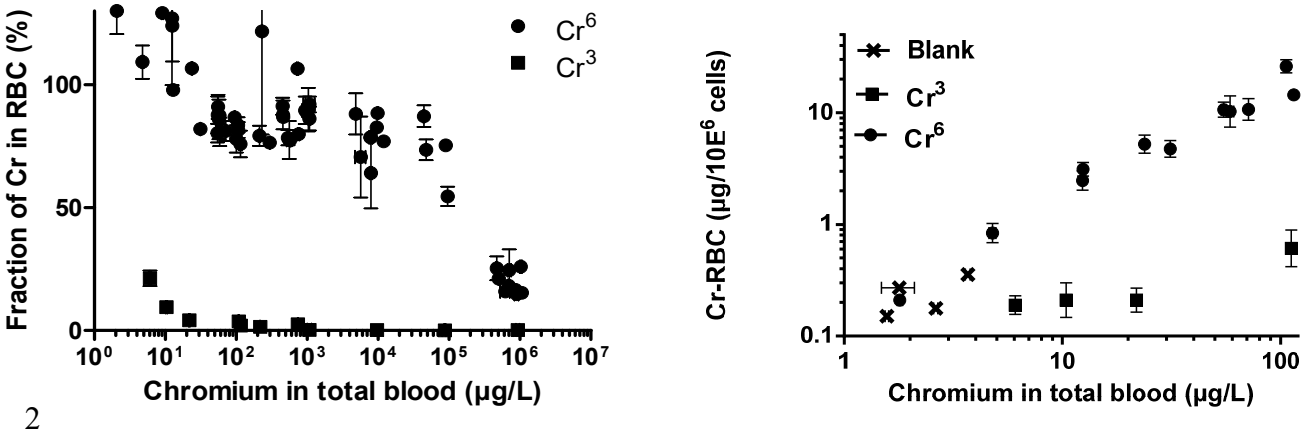
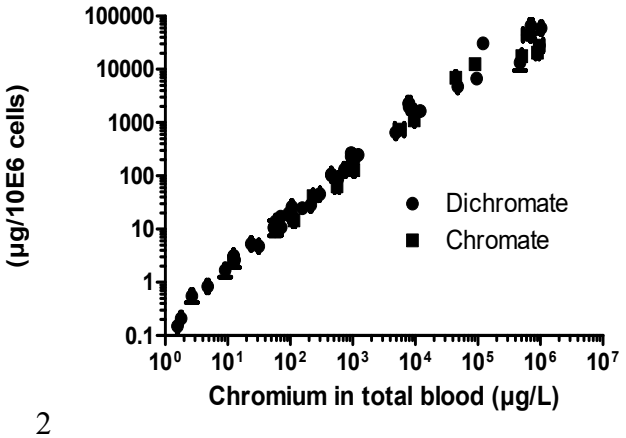


Fig. 1. Influence of chromium oxidation state on the accumulation of chromium in RBC. Incubation time of 40 minutes at room temperature. Each data point was derived from duplicate analyses.

A: Fraction of Cr accumulated in RBC.

B: Mass of Cr accumulated per million RBC, shown over the occupational range of Cr concentrations. “Blank”: no chromium added to blood. The slopes defined by the Cr(III) and Cr(VI) data are significantly different ($p<0.0001$).

A



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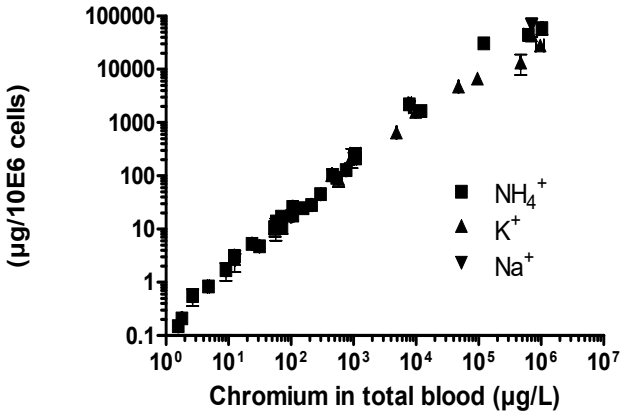

Fig. 2. Effect of chromium speciation on the accumulation of Cr in RBC according to the amount of chromium added to blood samples. Incubation time of 40 minutes at room temperature. Each data point was derived from duplicate analyses.

A: Effect of chromate and dichromate on the fraction of Cr(VI) accumulated in RBC. Statistically, there is no significant difference between the two slopes.
B: Effect of different counterions (NH_4^+, K^+ and Na^+) on the fraction of dichromate accumulated in RBC. Statistically, there is no significant difference between the slopes.

A B

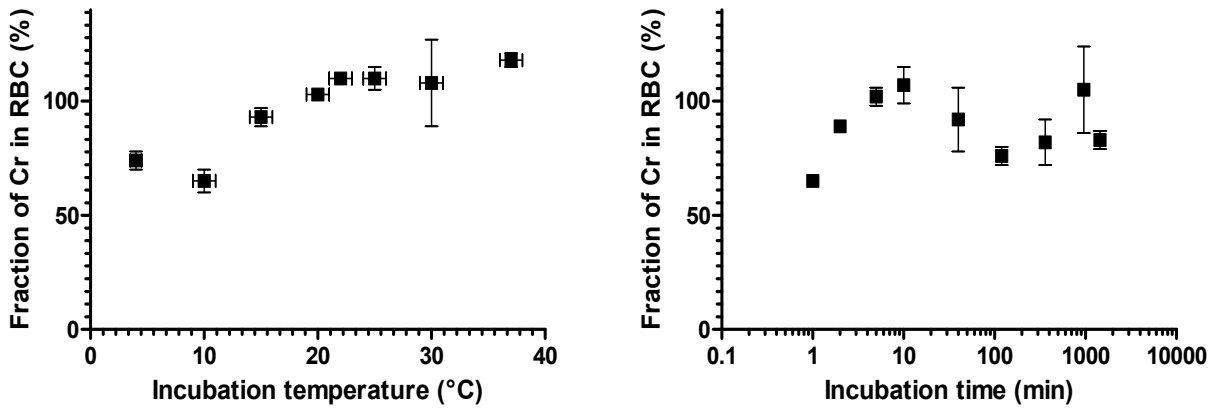

Fig. 3. Influence of incubation parameters (temperature (A) and time (B)) on the fraction of Cr(VI) accumulated in RBC. Each data point was derived from duplicate analyses.

A: Effect of incubation temperature. Incubation time of 40 minutes. 100 µg/L of Cr as $(NH_4)_2Cr_2O_7$ in 10 mL blood sample.

B: Effect of incubation time. 100 µg/L of Cr as $(NH_4)_2Cr_2O_7$ in 10 mL blood sample at room temperature.

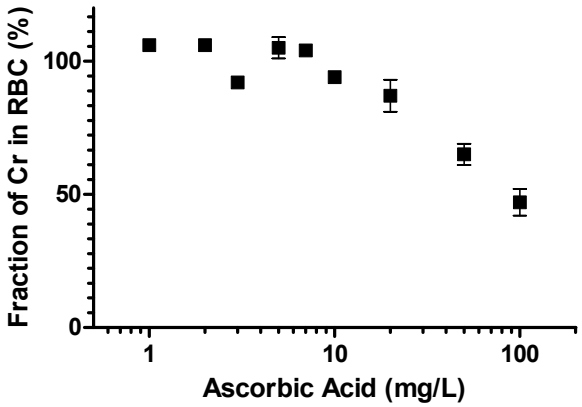

Fig. 4. Influence of AA on incorporation of hexavalent chromium in RBC. Incubation time of 40 minutes at room temperature. 100 µg/L of Cr as $(NH_4)_2Cr_2O_7$ in a 10 mL blood sample. Each data point was derived from duplicate analyses.

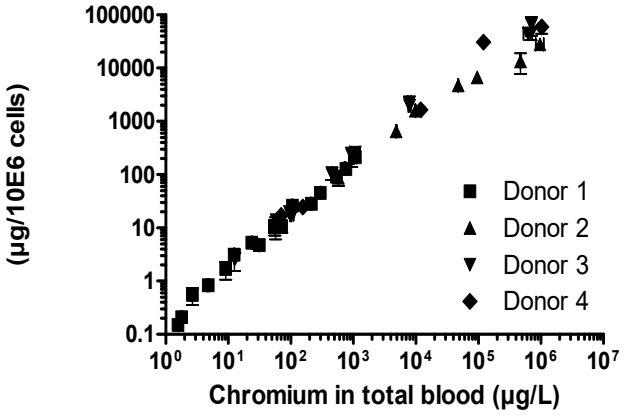

Fig. 5. Effect of blood donor. Incubation time of 40 minutes at room temperature. $(NH_4)_2Cr_2O_7$ in 10 mL blood sample. Each data point was derived from duplicate analyses. Statistically, there is no significant difference between the slopes defined by each donor.

Table 1. Instrumental settings for the determination of chromium in HNO_3 solution by GF-AAS.

Wavelength (nm)	234.9
Lamp current (mA)	10
Slit Width (nm)	0.5
Dry Temperature (°C)	300
Dry time (ramp/hold, s)	150/0
Ashing Temperature (°C)	20/10
Ashing time (ramp/hold, s)	50/30
Atomization Temperature (°C)	2600
Atomization time (ramp/hold, s)	0.8/2
Cleaning Temperature (°C)	2700
Cleaning time (ramp/hold, s)	2/0