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Comprehensive analysis of renal arsenic accumulation using images based on X-ray fluorescence at the tissue, cellular, and subcellular levels.

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Abstract

Exposure to arsenic (As) through drinking water results in accumulation of As and its methylated metabolites in several organs, promoting adverse health effects, particularly potential development of cancer. Arsenic toxicity is a serious global health concern, since over 200 million people are chronically exposed worldwide. Abundant biochemical and epidemiological evidence indicates that the kidney is an important site of uptake and accumulation of As, and mitochondrial damage plays a crucial role in arsenic toxicity. However, non-destructive analyses and in situ images revealing As fate in renal cells and tissue are scarce or almost non-existent. In this work, kidney tissue from exposed rats was analyzed by EDXRF (Energy dispersive X-ray fluorescence), micro-SRXRF (micro X-ray Fluorescence using Synchrotron Radiation), SRTXRF (SRXRF in total reflection condition), SEM-EDX (Scanning Electron Microscope in combination with EDXRF) and SRXRF-XANES (SRXRF in combination with X-ray Absorption near Edge Spectroscopy). Our results provide evidence of renal cortex distribution of As with periglomerular localization, co-localization of S, Cu and As in subcellular compartment of proximal tubule cells, mono-methylarsonous acid accumulation in renal cortex mitochondria, and altered subcellular concentration and distribution of other elements.

Keywords: bioaccumulation; arsenic; thioarsenicals; nephrotoxicity; imaging; XRF; chemical mapping; mitochondria

1. Introduction

Arsenic (As) is a naturally occurring metalloid classified as Class-A human carcinogen. The primary route of human exposure is through the consumption of contaminated water sources. Since over 200 million people are chronically exposed to concentrations above the World Health Organization's limit of 10 $\mu\text{g/L}$ (WHO), in 105 countries across 5 continents, As toxicity is a serious global health concern (Bundschuh et al., 2012; Shakoor et al., 2017). Following ingestion, As is efficiently absorbed in the gastrointestinal tract, binds to red blood cells and is distributed to the main organs throughout the body to undergo biotransformation to mono- (MMA^{III} , MMA^{V}), di- (DMA^{III} , DMA^{V}), tri-methylated (TMA^{III} , TMAO^{V}) and sulfur-containing metabolites (Herath et al., 2018; Khairul et al., 2017; Vahter 2002; Thomas et al., 2001). Then, chronic As exposure results in the accumulation of arsenic and its metabolites in several organs, inducing mitochondrial reactive oxygen species production, metabolic dysfunction, DNA damage and finally promoting health effects, particularly cancer (Aposhian and Aposhian, 2006; Lu Gamboa-Loiraa et al., 2017; Minatel et al., 2018; Yi et al., 2019). Consistently, animal models reveal arsenic accumulation in organs such as the brain, spleen, kidneys and liver, following chronic exposure through drinking water (Gailer et al., 2000; Hughes et al., 2003; Pérez et al., 2006 and 2008; Rubio et al., 2008; Ponomarenko et al., 2017; Soria et al., 2017). However, because arsenic is mainly eliminated in urine, kidneys and predominantly the renal cortex accumulate higher quantity of As than other organs, with proximal convoluted tubule damage (Rubatto Birri et al., 2010; Sabatha and Robles-Osorio, 2012; Khairul et al., 2017). Although there is abundant biochemical evidence supporting above data, non-destructive analyses and images revealing the As fate in renal tissues are scarce. A few examples anticipate that X-ray fluorescence spectrometry (XRF) allows to study accumulation and

distribution of As and other elements without destroying sample texture (allowing posterior analyses and re-evaluation studies), sometimes without pre-treatment, and spectral acquisition time is short (Pérez et al., 2006; Pérez et al., 2008; Rubio et al., 2008; Ponomarenko et al., 2017; Soria et al., 2017; Zhang et al., 2018). Direct images using X-ray fluorescence spectroscopy (XRF) can provide accurate and comprehensive picture of As bioaccumulation, which may contribute to further progress to alleviate arsenic-induced health effects. In this sense, images obtained by X-ray absorption spectroscopy (XAS) demonstrated the in vivo formation of seleno bis-(S-glutathionyl) arsinium ion $[(GS)_2AsSe]^+$, which was rapidly excreted via the bile into the intestine (Ponomarenko et al., 2017). This discovery has induced clinical trials for the development of chelation therapies.

The purpose of this study was to examine the disposition and metabolism of As and other elements in renal structures, tissues, cells and organelles from rats, after chronic exposure to arsenite. This was carried out by EDXRF (Energy dispersive X-ray fluorescence), micro-SRXRF (micro X-ray fluorescence using Synchrotron radiation), SRXRF-XANES (SRXRF in combination with X-ray absorption near-edge spectroscopy), SEM-EDX (Scanning Electron Microscope in combination with Energy dispersive X-ray fluorescence).

2. Methods and materials

2.1. Sample preparation

In order to avoid hormonal disturbances, each experiment included 10 adult (12-week-old) male Wistar rats. All experiments comply with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were approved by the Bioethics Committee of the Faculty of Medical Sciences of the National University of Cordoba,

Argentina, according to SAREM protocols (Web link:
<https://doi.org/10.1371/journal.pbio.1000412>).

The rats were randomly assigned to As and control study groups. The As group received drinking water containing 50 mg/L of As as NaAsO₂ during 60 days (2 mg/kg/day, for an average daily intake of 50 ml/animal, according to Rubatto Birri et al., 2010). The animals were fed on commercial animal diet (Cargill Inc., Co.) *ad libitum*. Drinking water and food were analyzed by conventional X-ray fluorescence spectrometry (Pérez et al., 2006), which gave a total arsenic level of less than 0.5 µg/g (limit of detection). After the administration of the last arsenic dose, the animals were given a 1-day rest and were sacrificed by decapitation. The kidneys were excised (without renal pelvis) and washed with NaCl 0.9%; the capsule was then removed and immediately processed according to the following protocols:

2.1.1. Subcellular Fractionation (all procedures were carried out at 0-4°C using ultra-pure reactive):

2.1.1.1. Cytosolic and non-soluble fractions separation: kidneys (both rat kidneys per sample, totaling 5 samples per group of control and As-treated rats) were immediately homogenized with ULTRA-TURRAX in Tris/HCl buffer (50 mM, pH 7.2) containing 20 µg/ml aprotinin as protease inhibitor. The homogenate was centrifuged at 100,000 g for 60 min. The resulting supernatants (SN) and the particulate fractions (PF) were collected in sterile Eppendorf tubes, lyophilized and immediately capped under nitrogen atmosphere. The tubes were stored in hermetic recipients under nitrogen atmosphere until their analysis by SRTXRF.

2.1.1.2. Mitochondria isolation was carried out according Rodriguez et al., (2007). Briefly: renal cortex (both rat kidneys per sample, totaling 5 samples per group of control and As-treated rats) were immediately cut in small fragments and treated with

proteinase K, washed with buffer and homogenized. The mitochondrial fractions were isolated by differential centrifugation and density gradient with sterile Percoll. The efficiency of isolation procedure was checked by quantifying the activity of mitochondrial enzyme Succinate dehydrogenase in each sub-fraction according to Pennington (1961). In order to preserve physicochemical properties, the pure mitochondria fractions were collected in sterile Eppendorf tubes, lyophilized and immediately capped under nitrogen atmosphere. The tubes were stored in hermetic recipients in a nitrogen atmosphere until their analysis by SRXRF-XANES.

2.1.2. Samples for EDXRF measurements: slices of 1 mm thickness were obtained with a ceramic scalpel, adhered onto Ultralene® film and lyophilized. The slices included renal cortex and medulla.

2.1.3. Samples for micro-SRXRF measurements: slices of 100 microns thickness were obtained on a cryostat, adhered into Ultralene® film and dried at 40°C by 1 min. The slices included renal cortex.

2.1.4. Samples for SEM-EDX analyses: fragments of 1 mm³ were obtained from the renal cortex, fixed in glutaraldehyde, dehydrated in acetone and included in Mollenhauer Mixture resin (Araldite-Poly/Bed® 812 mixtures, Polysciences Inc., catalog N° 02595-1). Ultra-fine slices of 0.1 µm were obtained and stained by the Microscopy Service of the University of Girona, Spain.

All samples for multielemental mapping by EDXRF, micro-SRXRF, and SEM-EDX were stored in hermetic recipients under nitrogen atmosphere until analysis. Biological material was transported with SENASA (Argentinian National Service for Agri-food Health and Quality, legal regulator in Argentina; where applicable) permission.

2.2. Multielemental mapping using Energy dispersive X-ray fluorescence (EDXRF) spectrometry

Mappings of X-ray fluorescence intensities in freeze-dried kidney slices were done at the IDAEA, Barcelona, Spain. EDXRF measurements was performed using a benchtop Fischer XDAL spectrometer (Helmut Fischer GmbH, Germany) with a 50 kV tungsten X-ray tube as radiation source. To ensure a quantitative analysis and the adequate combination of beam collimator and acquisition time, measurements were carried out under different conditions of beam collimation (0.2, 0.6, 1.0 and 2.0 mm) and measuring time (30-1000 s) (results in Fig. S1, Supplementary Material). Then we fixed the conditions for the rest of the experimental work at 0.6 mm of X-ray beam size and to keep the influence of geometry to a minimum, the counting time for each analytical point was fixed at 300 s. In order to reduce the background noise we fixed the use of a thick primary aluminum filter (1 mm). Under those conditions, the statistical error was at an acceptable level and the relative standard deviation of the measurements for Fe, Cu and As were around 1.5 %. Each sample was positioned in a motorized plate stage with a three-axis (X/Y/Z) remote controlled stage. A video microscope (magnification 100x) was used to precisely locate the points of reference for the beam incidence. To obtain maps of the element distribution on kidney slices, griddings of 10x10 or 20x20 points were programmed on the sample with steps of 0.6 mm in both directions of the grid. Incident beam impinged the sample surface at 90 degrees, and a detection system (SD detector) was placed at an angle of 45 degrees. All spectral data was automatically recorded for processing or re-evaluation if needed. Characteristic $K\alpha$ X-ray lines of elements were used for mathematical deconvolution and calculation of mass concentrations. Evaluation of the resulting EDXRF spectra was made using WinFTM software, version 6.19. Theoretical basis and practical applications of this software package are found in Roessiger and Nensel, 2006. Samples were analyzed by Fundamental Parameters approach (standardless mode), using generic organic matter

chemistry for Z-effective matrix correction, and by empirical calibration. For this last case, standards of cellulose spiked with 5, 25, 40, 100, 500 and 1000 $\mu\text{g/g}$ of Fe, Cu, and As were used for the instrumental calibration. Accuracy of the method was tested using organic certified reference material NCS DC73350 (leaves of poplar) from China National Analysis Centre Iron and Steel, Beijing, China. The potential of this configuration for analysis and elemental mapping has been previously demonstrated (Gallardo et al., 2016).

2.3. Multielemental measures using synchrotron radiation X-ray fluorescence spectroscopy (SRXRF)

The elements were measured by X-ray fluorescence spectroscopy at the XRF station of the Brazilian Synchrotron Light Laboratory (LNLS), (details of the XRF beamline can be found at <http://www.lnls.cnpem.br/linhas-de-luz/xrf-en/overview/>). Measurements were performed in air, samples were positioned with a three-axis (X/Y/Z) remotely controlled platform, and X-ray fluorescence spectra were collected using a Silicon Drift Detector (SDD). Cytosolic (SN) and non-soluble fractions (PF) were solubilized in 0.2% sodium dodecyl sulfate (SDS) in MilliQ water. Quantification was carried out by internal standardization by addition of Y to get a final 2 $\mu\text{g/ml}$ concentration, and 3 μl specimens were pipetted onto Ultralene® film. $K\alpha$ X-ray fluorescence intensities were measured in grazing incidence geometry in total reflection condition (SRTXRF) according to previously reported setup (Figuerola and Bongiovanni, 2007). The SRTXRF determinations were performed by exciting the samples with a white synchrotron spectrum for 200 seconds. For mapping of elements in freeze-dried kidney sections of 100 μm thick, the samples were set on the horizontal plane at 45 degrees from the incident beam to minimize scattering (conventional incidence geometry: 45-45 degrees), as previously reported (Pérez et al., 2006; Rubio et al., 2008; Rubatto Birri et

al., 2010; Pérez et al., 2014; Lamela et al., 2018). Samples were excited by a white beam, which was condensed to spot size of 20 μm in diameter using a glass monocapillary for multielemental mapping according to Pérez et al. (2016), and the counting time per point was of 10 seconds. The step size for mapping was 20 μm in both directions, covering an area of around 600 μm x 600 μm on renal cortex. Spectra were processed by PyMca software developed by the software group of the European Synchrotron Radiation Facility (ESRF) (Solé et al., 2007). Elemental concentrations were determined by the Fundamental Parameter method, calibrated by curves obtained with certified reference materials, SRM 1573A (tomato leaves) from NIST, IAEA-336 (lichen) from International Atomic Energy Agency, SRM 1577b (bovine liver) from NIST, and TORT2 (lobster hepatopancreas) from the National Research Council of Canada, Institute for National Measurement Standards.

2.4. Cellular arsenic distribution by Scanning Electron Microscope with Energy Dispersive X-ray Spectrometer (SEM-EDX)

Morphological and chemical characterization of microstructures in ultra-fine renal cortex slices was done at the Microscopy Service of the University of Girona, Spain (<https://www.udg.edu/ca/str/Aparells-tecniques-disponibles/Microscopia-i-Biotecnologia/Microscopia-electronica-de-rastreig-MER>). The electron microscopy images were obtained using a scanning electron microscope Zeiss DSM 960A, with voltage ranging 1 to 30 kV and EDX detection modes. The EDX detector (Bruker Nano XFlash Detector 5010) with a resolution of 129 eV (line Mn K α , 1000cps) is able to detect elements from atomic number 6 (carbon), allowing chemical characterization and elemental chemistry calculations using classical ZAF (6-interactions) methodology for matrix correction. Apparent concentration and percent composition by element and

atom were obtained applying an acceleration voltage of 12 kV. The limits of detection (LOD) for As were 0.32, 0.22 % and 0.04 %, respectively.

2.5. Arsenic speciation by XANES in renal mitochondria

The mitochondria samples were solubilized in 0.2% sodium dodecyl sulfate (SDS) in MilliQ water, and 2 μ l specimens (or arsenic standards) were pipetted onto Ultralene® film. The species of accumulated arsenic in renal mitochondria was analyzed by X-Ray Absorption Near Edge Spectroscopy (XANES) at the XRF station of the Brazilian Synchrotron Light Laboratory (LNLS). The excitation energy was selected using a Si(111) channel-cut crystal monochromator and calibrated by defining the first derivative peak of Au foil spectrum 11919.0 eV (Au L3-edge). All spectra were recorded in fluorescence mode. The excitation energy was tuned across the As K-edge ($E_0=11867$ eV) from 11800 eV to 12000 eV. Spectra for the pre-edge region were recorded from 11800 to 11854 eV using step sizes of 2 eV, 0.5 eV for the edge and 2 eV above 11947.5 eV. Counting times were 3 s per point on the pre-edge and post-edge regions, and 9 s for the edge. The main features of the XANES spectra from the sample were qualitatively compared with those obtained from 100 μ g/ml solutions of the following analytical standards: As^{III} (Arsenic trioxide, catalogue N° 72718 Sigma-Aldrich; <http://www.sigmaaldrich.com/argentina.html>), As^V (Arsenic pentoxide, catalogue N° 76686 Sigma-Aldrich), MMA^{III} (monomethyl arsenic diiodide, catalogue N° AR60013, Argus Chemicals SRL, <http://www.arguschem.com/catalogue/arsenic-standards/>), MMA^V (monomethylarsonic acid disodium salt, catalogue N° AR60009, Argus Chemicals SRL) and DMA^V (Dimethylarsinic acid, catalogue N° PS51 Sigma-Aldrich). Data analysis was performed using the Athena software in the computer package IFEFFIT (Ravel and Newville, 2005). The background was corrected by fitting a first-

order polynomial to the pre-edge region between -80 and -20 eV relative to E_0 and a quadratic normalization function between 45 and 150 eV above E_0 .

2.6. Statistical analysis

Five samples of cytosolic (SN) and non-soluble (PF) fractions per group of control and As-treated rats were analyzed by SRXRF. Five samples of mitochondria per group of control and As-treated rats, as well as 3 samples of each standard, were analyzed by XANES. Data were expressed as an average of values obtained in each determination. Multielemental mapping by EDXRF was performed in five renal slices from As-treated rats. (One slice per rat was analyzed). Multielemental mapping by SRXRF was performed in five renal cortex slices from As-treated rats (one slice per rat was analyzed). Colored scales for graphed elements are represented as an average of the results. Five electron-dense inclusions were analyzed by SEM-EDX in each ultra-fine slices of renal cortex from three As-treated rats (one slice per rat was analyzed. Data were expressed as an average of values obtained in each determination. The comparison of the T-test was used to evaluate the significant differences between averages. The differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Multielemental mapping using EDXRF

In Fig. 1, the distribution of major detected elements in the kidneys of As-treated rats are showed. The results obtained by EDXRF using benchtop equipment are consistent with those obtained previously by SRXRF with a beam of $300\ \mu\text{m} \times 300\ \mu\text{m}$ (Pérez et al., 2006), showing that renal cortex was the principal kidney region of As accumulation. Since blood hemoglobin is the principal carrier of Fe in the body, the distribution pattern of Fe fingerprints coincides with vascular system. Therefore, the renal zones with high Fe levels correspond to the regions with high irrigation or major

kidney vessels. In this regard, high Fe label was observed in the region located between the renal cortex (Cx) and renal medulla (M), where the arcuate arteries are placed. Each arcuate artery supplies blood to several interlobular arteries that feed the afferent arterioles of glomeruli. Indeed, glomeruli are distributed in renal cortex, where Fe concentration was also high, while Fe label was minor in renal pyramid (P) and low in the calix (C). This corresponds with few vessels of small caliber in those areas with increase of urine conducts. Similar Fe distribution was observed in control animals. In Fig. 1, it is observed that in the area occupied by the arcuate arteries the content of Fe (250-350, $\mu\text{g/g}$) is slightly greater than the one found for As (150-250, $\mu\text{g/g}$). Since dimethylarsinous acid (DMA^{III}) is the arsenic species that binds to hemoglobin with a stoichiometry of 1:1 (Lu et al., 2007 and 2008), the image obtained by EDXRF is in agreement with those biochemical results, showing that a significant fraction of hemoglobin is bound to As. According to Lu et al. (2007 and 2008) As in the blood would be mainly DMA^{III} . On the other hand, the amount of As (350-500, $\mu\text{g/g}$) is higher than Fe (250-350, $\mu\text{g/g}$) in the renal cortex. In this area, there are large numbers of glomeruli and convoluted tubules. This result suggests that As is being retained by another target besides hemoglobin in renal cortex. Likewise, Cu showed a similar distribution to that of As in the renal cortex, with a high concentration (up to 250 $\mu\text{g/g}$), but it was almost undetected in other irrigated zones such as the medulla because Cu is not bound to hemoglobin. In addition, unlike Fe whose concentration and distribution were not altered by chronic exposure to As, Cu concentration in renal cortex increased eight times in comparison to controls. Similar results were previously reported (Pérez et al., 2006). The distribution of As and Cu along glomeruli and tubules was analyzed by micro-SRXRF, with better spatial resolution.

3.2. Multielemental mapping using micro-SRXRF

Multielemental mapping with higher spatial resolution was obtained using a synchrotron beam allowing a spatial resolution of 20x20 μm per pixel. This is an appropriate resolution since the diameter of proximal tubules is around 60 μm and 200 μm the glomerulus. Fig. 2 shows two zones of high Fe concentration pointing at glomeruli. The image suggests that the upper glomerulus was cut in half by the cryostat while the lower one was cut tangentially. The Fe label is minimal around glomeruli. Fig.2 also shows that the pattern of distribution of As and Cu are quasi identical in renal cortex. Both elements distribute around glomeruli, where proximal convoluted tubules are most numerous. This result is in agreement with in vitro and in vivo studies, suggesting that proximal convoluted tubules are one of the primary targets of As intoxication (among others: Kenyon et al., 2008; Peters et al., 2014). Studies indicate that several membrane transporters in cells of proximal convoluted tubules may mediate the uptake of As^{III} and MMA^{III} , inducing oxidative stress and genetic and epigenetic alterations that result in proneoplastic changes (Orr and Bridges, 2017). In order to investigate the subcellular localization of As, ultra-micro analysis was conducted by SEM-EDX.

3.3. Cellular arsenic distribution by SEM-EDX

This methodology can obtain data on both the distribution of elements and the biological ultrastructure of the analyzed location at the same time. In Fig. 3, an image obtained with the optic microscope was included (right). This image illustrate that proximal tubules (PT) are easily distinguishable from distal tubules (DT) or glomeruli (Glom). Both tubules are formed by a simple epithelium. However, proximal tubules present reduced lumen. Thus, only proximal tubules were analyzed by SEM-EDX. Fig. 3 (left and middle) shows that numerous electron-dense inclusions are distributed within the cells of this tubular epithelium of As-treated rats. Other authors have previously

described formation of As-induced inclusions in mitochondria of human and mouse urothelium (Dodmane et al., 2014). However, the composition of the inclusions in the urothelium is not known. When electron beam spot was focused on renal inclusions, we were able to analyze their composition by EDXRF. The results indicate that the elements in the inclusions are (elemental composition is expressed as percentage) C (69.6 ± 1.5), O (18.5 ± 2.6), P (0.2 ± 0.1), S (2.6 ± 0.9), Cl (3.9 ± 1.4), Cu (3.0 ± 1.1), and As (2.2 ± 0.5). This observation corroborates that As is incorporated and accumulated by cells of proximal tubules. However, the most significant result is that for the first time subcellular compartmentalization of As can be visualized in these cells. Interestingly, S and Cu are also compartmentalized together As in an atomic proportion 3:2:1, respectively. Thus, the presence of thioarsenicals, which has been described in kidney, cannot be ruled out (Herath et al., 2018). Taking into account that: a) Cu is an essential component of the mitochondrial inner membrane complexes, b) As accumulation has been shown in renal and urothelial mitochondria (Vahter and Marafante, 1989; Dodmane et al., 2014), c) mitochondrial rhodanese is involved in the formation of methylated thioarsenate compounds (Shimoda et al., 2015), d) exposure to As induced formation of inclusions in mitochondria (Dodmane et al., 2014) and that e) mitochondria appear to be important targets of As-induced cellular toxicity, the results by SEM-EDX suggest that the renal accumulated As could be associated with Cu and S in inclusions inside mitochondria from cells of proximal tubules.

3.4. Multielemental distribution in subcellular fractions using SRXRF

3.4.1. Elemental concentration in cytosolic and non-soluble fractions: To analyze the effect of As consumption on subcellular distribution of elements in renal tissue, cytosolic and non-soluble fractions were obtained. The cytosolic fraction corresponding to supernatant (SN) contains soluble molecules from cytosol and extracellular

compartments, tubular lumens and possibly from some crashed organelles. This means the particulate fraction (PF) contains larger non-soluble components such as membranes, fibers and organelles. After As treatment, most of the detected elements maintain this concentration and distribution. However, the results shown in Fig. 4 indicate chronic ingestion of As alters the concentration and distribution of some elements. Indeed, As and Si were detected in SN and PF after As treatment, but As concentrations were higher in SN (240 $\mu\text{g/g}$) than in PF (150 $\mu\text{g/g}$). On the other hand, K concentration was higher in PF than in SN in control kidneys, and this could be related to electrostatic interaction of K^+ (and Na^+ , but this element is not detected by LNLS Synchrotron) with phospholipids of membranes. However, after As treatment, K concentration increased by a factor of eight in the SN becoming higher than PF. As described by Baur et al. (1975), the K leakage is related to cell damage, and this is in agreement with previous results showing tubular damage induced by As treatment (Rubatti Birri et al., 2010). On the other hand, high As and S concentrations in SN could be related to As-hemoglobin interaction in rat blood (Lu et al., 2007 and 2008), as well as the presence of arsenical or thioarsenical metabolites and their glutathione (GSH) conjugates, which may be exported into the tubular lumen and excreted by urine to protect the body (Liu et al., 2006; Orr and Bridges, 2017; Herath et al., 2018; De Loma et al., 2019). On the contrary, Cu concentration increases by a factor of seven in the PF becoming higher than in the SN. This is in agreement with results showed above which suggest that As treatment induce Cu and As mitochondrial accumulation. Accordingly, a GSH-thioarsenicals-Cu interaction in the kidney cannot be ruled out because arsenite and thioarsenicals both have a high propensity to react with glutathione. Furthermore, strong interactions between Cu and thioarsenite have been observed in natural or synthetic chalcogenides, such as enargite (Cu_3AsS_4 , Clarke and Helz, 2000) or

chalcogenidoarsenate anions containing Cu ($\text{Cu}_2\text{AsS}_3^-$, Kromm et al., 2010). However, unlike what happens in the liver, where Se forms seleno bis-(S-glutathionyl) arsinium ion $[(\text{GS})_2\text{AsSe}]^-$ and is rapidly excreted via bile into the intestine (Gailer et al., 2000; Ponomarenko et al., 2017), we speculate that GSH-thioarsenicals-Cu could be, at least in part, retained in the tubular mitochondria, accounting for the increase of Cu in PF although this speculation is yet to be demonstrated. Since toxicity of As is dependent on its oxidation state, the As species retained mitochondria were analyzed by SRXRF-XANES.

3.4.2. Arsenic speciation by SRXRF-XANES in renal mitochondria: Abundant biochemical information indicate that the mitochondria is the organelle most affected by monomethylarsonous acid (MMA^{III}), and this is implicated in the pathogenicity of arsenic exposure (Petrick et al., 2000; Styblo, et al., 2000; Thomas, et al., 2001; Liu et al., 2005; Naranmandura et al., 2011; Pace et al., 2016; Minatel et al., 2018). However, it has been described that the uptake of arsenic compounds appears to be highly dependent upon cell type and arsenic species (Vahter and Marafante, 1989; Dopp et al., 2010; Pace et al., 2016). Here, mitochondria were isolated from renal cortex of rats exposed to As^{III} to elucidate the types of As retained species. XANES spectra corresponding to the standards and mitochondrial are presented in Fig. 5. The absorption edge energy of mitochondria was 11871 eV, and this value corresponds to MMA^{III} and agrees with XANES edge position reported in the literature (Smith et al., 2005). An additional contribution is observed as deformation in mitochondria XANES spectra at 11867 eV (Fig. 5). This XANES edge position has been described for trithioarsenite (Price et al., 2010). We do not have standard to corroborate this hypothesis. However, all evidence here showed, obtained by X-Ray Fluorescence Spectrometry methodologies, suggests the presence of thioarsenic compounds in

mitochondria from renal tubules. Further studies would be interesting because the thiolated arsenic compounds are more toxic than their counterparts without sulfur (Herath et al., 2018).

4. Conclusions

In summary, the results indicated that: 1) rats that took arsenite for 60 days showed an accumulation of As and Cu in the renal cortex; 2) As and Cu accumulate around the glomeruli; 3) Two-thirds of the accumulated As is soluble, in agreement with the excretory/detoxifying function of the kidney; 4) As treatment alters the concentration and subcellular distribution of other elements; 5) As, Cu and S co-localize in subcellular compartments of tubular cells, suggesting a possible association; 6) a relevant part of the As accumulated in the mitochondria is MMA^{III} .

Thus, the result of our determinations suggests that the chain of events that lead to nephrotoxicity could be related to the preferential uptake of MMA^{III} in tubular mitochondria.

Arsenicosis has been known for more than 100 years (Ayerza, 1917; Goyenechea, 1917); however, there is still no antidote to prevent its harmful effects. Considering the millions of people who consume toxic amounts of arsenic in their drinking water or food, the fate of arsenic at tissue, cellular and subcellular levels and its highly toxic metabolite MMA^{III} are data that acquire great medical relevance. X-ray fluorescence spectrometry (XRF) is a fast analytical method with an atomic resolution that requires minimal sample preparation; it provides useful information for biomedical and toxicological studies, and offers unparalleled advantage of direct measurement of the distribution of elements at tissue, cellular and subcellular level. The data of spatial distribution and accumulation of toxic elements and dyshomeostasis of essential elements, combined with biochemical information, are of great importance because they

can help to understand the processes that lead to diseases and can contribute in the development of better therapies. The identification of the elemental species involved in pathological processes is also a critical step towards understanding the toxicity of elements such as As, with rich redox biochemistry and complex metabolization pathways.

We hope that these data obtained by XRF can be of great value in the field of toxicological research, histopathology, diagnosis and the development of therapies to fight against the nephrotoxicity of arsenic.

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LEGEND OF FIGURES

Fig. 1. Spatial distribution of As, Cu and Fe concentrations determined by EDXRF in kidney slices from As-treated rats. Concentration in ppm ($\mu\text{g/g}$) according to scale. The analyzed area is indicated with a rectangle in the image of the renal slice (top left). Cx: renal cortex, M: renal medulla. P: renal pyramid. C: calix. The figure shown a representative result, however, concentration scale was adapted to the average of the five determinations.

Fig. 2. Spatial distribution of Fe, As and Cu concentrations determined by μSRXRF analysis for renal cortex slices from As-treated rats. Concentration in ppm ($\mu\text{g/g}$) according to scale. The figure shown a representative result, however, concentration scale was adapted to the mean of ten determinations.

Fig. 3. Representative photomicrographs of the periglomerular zone in after chronic As exposure for 60 days: Left: image obtained by SEM-EDX highlighting electron-dense inclusions. Middle: same left image where some proximal tubules were delineated for better visualization. Right: renal cortex stained with H/E (magnification 400x); asterisks denote tubular alterations; PT: proximal tubules (small lumen); DT: distal tubule; Glom.: glomeruli.

Fig. 4. Effect of As consumption on that subcellular distribution of elements in renal tissue. The fluorescence intensity of each element was determined by SRTXRF in the supernatant (SN) and the particulate fraction (PF) fractions obtained by 100,000g centrifugation. Then SN/PF ratios were calculated for kidneys of control and As-treated

rats. The Cl was omitted because homogenate was prepared in Tris/HCl buffer. The resulting values were plotted. Values >1 indicate major concentration in SN than P. * denote significant difference with respect to control.

Fig. 5. XANES spectra at arsenic K-edge obtained in mitochondria fraction and arsenic standards: As^{III} , MMA^{III} , MMA^{V} , DMA^{V} , and As^{V} . The final XANES spectra were processed after background subtraction and normalization to the post-edge intensity. Results near arsenic K-edge are shown in the figure. The dotted vertical line corresponds to MMA^{III} white line position (11871 eV). The values are the mean of four determinations in five mitochondria samples (five rats) or standards. The spectra are displaced vertically for clarity.

LEGEND OF FIGURES IN SUPPLEMENTARY MATERIAL

Fig. S1. Relative standard deviation (RSD as %) of EDXRF analysis in kidney slices from As-treated rats. RSD was obtained for As in different instrumental set up.

Fe

As

Cu

CRIP T

SN/PF ratios

SN/PF ratios

Highlights

The distribution of As in renal tissue was analyzed without pre-treatment

SRXRF and SEM-EDX allowed corroborate co-location of Cu and As in proximal convoluted tubules

For the first time, the compartmentation of arsenic in the renal tubules is shown in images.

The most toxic species of As MMA^{III}, accumulates in mitochondria

X-ray Fluorescence Spectroscopy provides an accurate and high-resolution picture of As bioaccumulation