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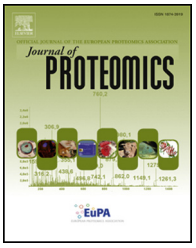

A combination of metallomics and metabolomics studies to evaluate the effects of metal interactions in mammals. Application to *Mus musculus* mice under arsenic/cadmium exposure☆

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ABSTRACT

Arsenic and cadmium are toxic metals of environmental significance with harmful effects on man. To study the toxicological and biochemical effects of arsenic/cadmium in mammals a combined metallomic and metabolomic approach has been developed, complemented with the measurement of biochemical parameters in blood and histopathological evaluation of liver injury in mice *Mus musculus* under exposure to both xenobiotics. Size-exclusion chromatography (SEC) was combined with affinity chromatography (AF) and ICP-MS detection using species unspecific isotopic dilution analysis (SUID) to characterize the biological effects of As/Cd on selenium containing proteins in the bloodstream of exposed mice. On the other hand, both direct infusion mass spectrometry (DIMS) and gas chromatography–mass spectrometry (GC–MS) provided information about changes in metabolites caused by metals. The results show that As/Cd exposure produces interactions in the distribution of both toxics between organs and plasma of mice and antagonistic interactions with selenium containing proteins in the bloodstream. Interplay with essential metabolic pathways, such as energy metabolism and breakdown of membrane phospholipids were observed, which are more pronounced under As/Cd exposure. In addition, heavy metal and metalloid causes differential liver injury, manifested by steatosis (non-alcoholic fatty liver disease, NAFLD) and infiltration of blood cells into the space of Disse.

☆ This article is part of a Special Issue entitled: Environmental and structural proteomics.

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Biological significance

This work presents new contributions in the study of arsenic/cadmium interactions in mice *Mus musculus* under controlled exposure. With the combination of metallomic and metabolomic approaches the traffic of As and Cd from liver to kidney by means of blood was observed and excretion of As (as arsenic metabolites) or Cd (as MTCd) is inhibited with the simultaneous administration of As/Cd, and these toxic elements have important influence in the levels of seleno-proteins in the plasma. In addition, the metabolomic approach reveals inhibition of different metabolic cycles such as tricarboxylic acid and phospholipid degradation that causes membrane damage and apoptosis that is histopathologically confirmed.

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1. Introduction

Arsenic (As) and cadmium (Cd) are important inorganic co-pollutants in the environment, which are the origin of numerous environmental issues. Biological systems are exposed to environmental complex ecosystems where the chemical species of the elements may interact with synergistic or antagonistic effects, and have to be considered in relation to the metabolic processes involved [1]. In addition, these metals are not biodegradable and have a long life in the environment. Accumulation of these toxic metals/metalloids in ecosystems is a major source of human exposure and hence a threat to human health, mainly As and Cd, which are by-products from processing other metals, leading to common exposure in industrial settings. The biochemical effects of independent exposure to As and Cd have been extensively studied in experimental animals [2–5], however, the biological response of mammals under simultaneous exposure to both toxicants has been poorly studied. As a result, the toxicological effects provoked by arsenic and cadmium administration remain still unclear. Additionally, in experimental systems, arsenic and cadmium exhibit a great influence on metabolic cell functions [4,6]. There are evidences about the interaction of As/Cd in rats, which is reflected in changes in different biomarkers assays [7]. These authors report that combined exposure to As/Cd is more damaging than separate exposure to each elements, inducing lipid peroxidation and both glutathione and metallothionein up-regulation.

In this sense, to obtain a representative information about changes in metabolites caused by complex metal exposure, *-omics* methodologies have been proposed as a good alternative [2,4]. Metallomics is a relatively new field related to metal-biomolecule expression and identification in biological systems, which represent a more than 30% of molecules in cells. In metallomics metals are used as markers or tags to track these molecules in complex biological matrices [8]. These approaches require the use of high sensitivity atomic detectors mainly ICP-MS [9], generally coupled to a chromatographic module (in single or multidimensional arrangements), and mass spectrometry for parallel biomolecule identification in an integrated workflow [9,10]. For this reason, metallomics provides a good alternative to deep insight into the fate of elements in exposed organisms to metals, and provides information about metals trafficking, interactions and homeostasis [11]. On the other hand, metabolomics is based on the comprehensive evaluation of metabolites involved in different metabolic processes in organisms, considering the metabolome as the entire cellular set of endogenous low molecular mass biomolecules (typically <1000 Da) [12]. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are major analytical tools used in metabolomics approaches [13,14]. Nevertheless, the performance of DIMS on biological fluids or tissues from mice under metal exposure has proved to be a good choice for this purpose [4].

In this work, a metallomic approach based on SEC-ICP-MS has been used to achieve a better understanding of the function, detoxification processes, interactions and regulation of metals in laboratory mouse *Mus musculus* under controlled exposure to arsenic and cadmium. Additionally, 2D-SEC-AF-SUID was performed to quantify selenium containing proteins in mice plasma with ICP-qMS as multielemental detector. On the other hand, intended to get as much metabolic information as possible, plasma and liver from these animals, after exposure to metals during 12 days, were also studied using direct infusion high-resolution mass spectrometry (DI-ESI-QqQ-TOF-MS). Statistical analysis of the results allowed us to compare the different metabolic profiles, establishing the metabolites altered by the presence of these contaminants. In addition, several metabolites were quantified by gas chromatography–mass spectrometry (GC–MS) in plasma from mice. Finally, the study has been complemented with the measurement of conventional biochemical parameters in blood and the histopathological study of liver mice.

2. Material and methods

2.1. Instrumentation

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770) was used for solid tissue disaggregation. Disaggregated tissues were subsequently disrupted with a glass/teflon homogenizer. The extraction was followed by ultracentrifugation with an ultracentrifuge Beckman model L9-90K (rotor 70 Ti). Polycarbonate bottles of 10 ml with cap assembly (Beckman Coulter) were used for this purpose. A microwave oven (CEM Matthews, NC, USA, model MARS) was used for the mineralization of extracts.

Trace elements and heteroelement-containing biomolecules were analyzed with an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan) equipped with an octopole collision/reaction cell. Chromatographic separations were performed by using a Model 1100 HPLC pump with detector UV (Agilent, Wilmington, DE, USA) as delivery system.

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Metabolomic experiments were performed in a mass spectrometer QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) by using the electrospray (ESI) source. The parameters for QqQ-TOF system were optimized to obtain the higher sensitivity with minimal fragmentation of molecular ions, both in positive and negative ionization mode. To acquire MS/MS spectra, nitrogen was used as collision gas.

Gas chromatographic analysis was performed in a Trace GC ULTRA gas chromatograph coupled to an ion trap mass spectrometer detector ITQ900, both from Thermo Fisher Scientific, using a Factor Four capillary column VF-5MS 30 m × 0.25 mm ID, with 0.25 μm of film thickness (Varian).

Blood activity of alanine transferase, alkaline phosphatase, amilase, lipase and aspartate transferase, and concentrations of bilirubin, albumin, ferritin, LDL, HDL, triglycerides and creatinine were determined by using an automated analyzer (Selectra Junior Spinlab 100, Vital Scientific, Dieren, Netherlands; Spinreact, Girona, Spain) according to the manufacturers' instructions.

2.2. Standard solutions and reagents

All reagents used for sample preparation in the metallomic approach were of the highest available purity. Phenylmethanesulfonyl fluoride (PMSF) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (BioUltra grade, >98%) were obtained from Sigma-Aldrich (Steinheim, Germany).

Standards used for mass calibration of analytical SEC columns (mass range 70–3 kDa) were: ferritin (440 kDa) (purity 95%), bovine serum albumin (67 kDa) (purity 96%), superoxide dismutase containing Cu and Zn (32 kDa) (purity > 70%), myoglobin (14 kDa) (purity > 98%), metallothionein I containing Cd, Cu and Zn (7 kDa) (purity > 95%) and arsenobetaine (179 Da) (purity > 98%). All these reagents were purchased from Sigma-Aldrich (Steinheim, Germany). The mobile phase used in SEC was 20 mM ammonium acetate (Suprapur grade) purchased from Merck (Darmstadt, Germany), which was prepared daily with ultrapure water (18 MΩcm) from a Milli-Q system (Millipore, Watford, UK). The pH was adjusted at pH 7.4 with ammonia solution, this later prepared by dilution of 20% (w/v) ammonia solution (Suprapur, Merck) with ultrapure water. The void volume was determined by using blue ferritin (440 kDa).

Human serum certified reference material BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Standard solutions of 1000 mg L^{-1} of Se stabilized with 5% (v/v) nitric acid Suprapur and of 1000 mg L^{-1} of Br- stabilized with 5% (v/v) nitric acid Suprapur were purchased from Merck (Darmstadt, Germany). Enriched ^{74}Se and ^{77}Se were obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder and it was dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to volume with ultrapure water.

All the solvents used in sample preparation for metabolomic study of liver tissue and plasma were of HPLC-grade. Methanol and chloroform were purchased from Aldrich (Steinheim, Germany), while dichloromethane and formic acid were supplied by Merck (Darmstadt, Germany).

Derivatizing agents, methoxylamine hydrochloride and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS), were obtained from Sigma-Aldrich. Alanine, valine, isoleucine, proline, glycine, serine, threonine, glutamic acid, phenylalanine, fructose, galactose, glucose, tyrosine, tryptophan, urea, aspartic acid, glutamine, cholesterol, α-ketoglutarate, isocitric acid, citric acid, lactic acid and uric acid were purchased from Sigma-Aldrich to be used as standard substances in gas chromatography quantification.

2.3. Animal handling

M. musculus (inbred BALB/c strain) mice were obtained from Charles River Laboratory (Spain). Mice 7 weeks of age were fed ad libitum with maintenance pellets deficient in metals content. The animals were allowed to acclimate for 5 days with free access to food and water under controlled condition (temperature (25–30 °C) and a 12 h light–dark cycle) prior to start exposure experiment. For the experiment exposure, a total of 64 *M. musculus* mice were divided into four groups (16 mice per cage): control group (*CONTROL GROUP*), group exposed to arsenic (*As GROUP*), group exposed to Cd (*Cd GROUP*) and finally, group simultaneously exposed to As and Cd (*As/Cd GROUP*).

Arsenic (As_2O_3) and cadmium ($CdCl_2$) were orally administrated by using an oral gavage for mice. The control group was treated with 100 μL of 0.9% NaCl. In the case of arsenic, daily dose was 3 mg/kg of body weight and per day and for cadmium 0.1 mg/kg of body weight and per day both together in a dose of 100 μL. *M. musculus* mice were sacrificed after the sixth day of the beginning of the experiment (8 mice in each group) and 12nd day of the experience to evaluate the effect of exposure conditions and diet.

Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, dissected by using a ceramic scalpel and finally the organs transferred rapidly to dry ice. In parallel a portion of each liver was reserved for the histological assessment. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% NaCl solution, frozen in liquid nitrogen and stored at −80 °C until their use for extract preparation. Plasma collection from five mice of each group was carried out by centrifugation (4000 *g*, 30 min, 4 °C), after addition of heparin (ANTICLOT) as anticoagulant for separation into plasma and red blood cells (RBCs). In addition, 10 mg of 100 mM of PMSF and 100 mM of TCEP mixture were added as proteases inhibitor and reducing agent, respectively, for metallomic studies. On the other hand, three samples of blood without any anticoagulant were used to the measurement of biochemical parameter. Mice were handled according to the norms stipulated by the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

2.4. Measurement of the clinical parameters in blood and histopathology in liver from mice under As/Cd exposure

Blood activity of alanine transferase, alkaline phosphatase, amilase, lipase and aspartate transferase and concentrations of bilirubin, albumin, ferritin, LDL, HDL, triglycerides and creatinine were determined. Standard controls were run before each determination, and the values obtained for the

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different biochemical parameters were always within the expected ranges. The intra-assay variability of biochemical tests was relative to 12 repeated determinations of the control serum in the same analytical session, whereas inter-assay variability for each parameter was calculated on the mean values of control sera measured during 6 analytical sessions. Both biochemical and histological examinations were utilized to assess liver injury.

2.5. Determination of total metals in plasma, liver and kidney

First of all, individual organs were disrupted by cryogenic homogenization. For total metal determination, three samples of plasma, pulverized livers and kidneys of mice from each group were exactly weighed (100 mg) in 5-ml microwave vessels and 500 mg of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v) was added. After 10 min, the PTFE vessels were closed and introduced into the microwave oven. The mineralization was carried out at 400 W from room temperature ramped to 160 °C for 15 min and held for 10 min at this temperature. Then the solutions were made up to 2 g with ultrapure water and the metals analyzed by ICP-MS. The element Rh was added as internal standard (1 ng g^{-1}). All the analyses were performed by using two replicates of each sample, using 5 mice per group.

2.6. Metallomic approaches based on ICP-MS detection for analysis of plasma, liver and kidney extracts of mice (M. musculus) under As/Cd exposure

Pools of organs from male mice of different groups of exposure were treated following a procedure described elsewhere [11] for later application of size exclusion chromatography with inductively coupled plasma mass spectrometry and octopol reaction system (SEC-ICP-ORS-MS). On the other hand, to avoid changes in selenium species, the samples were directly injected into the column, without prior dilution to evaluate the effects of cadmium in selenium containing proteins by in series two-dimensional size exclusion and affinity high performance liquid chromatography with ICP-MS detection (2D/SEC-AF-ICP-ORS-MS [15]. The fractionation of selenium containing proteins by two-dimensional chromatographic separations, based on SEC prior to the use of a double affinity column, was carried out following a procedure described elsewhere [15].

The quantification of selenium containing proteins and selenium-metabolites in the different chromatographic peaks was carried out by post-column species-unspecific isotopic dilution (SUID) analysis as described by C. Sariego-Muñíz et al. [16]. The intensity of different Se isotopes and polyatomic interferences were converted to mass flow chromatogram for the quantification of selenium species in plasma and serum samples. Dead time correction was carried out by using the procedure described by F. Vanhaecke et al. [17], which results in 47 ns in this study. Mathematical treatments were applied to correct BrH^+ and SeH^+ polyatomic interferences. Mass bias corrections were applied by using the $^{78}Se/^{74}Se$ and $^{80}Se/^{74}Se$ isotope ratios, calculated (exponential mode) as previously described by J. Ruiz-Encinar et al.[40]. Finally, online dilution equation was applied to each point of the chromatogram and the amount of selenium in each chromatographic peak calculated by using the Origin 8.5.1 software (Microcal Software Inc., Northampton, MA, USA).

2.7. Metabolomic study of plasma of mice (M. musculus) under As/Cd exposure by DI-ESI(±)-QTOF-MS

For metabolomic analysis, metabolite extraction from individual plasma was carried out in a two-step approach following a procedure described elsewhere [4]. The polar and lipophilic extracts were reconstituted to 200 μL with (1:1) chloroform/water mixture before the analysis by ESI-MS. For DI-ESI(±)-QTOF-MS of plasma samples, proteins were removed from blood plasma by adding 400 μL of 1:1 methanol/ethanol mixture to 100 μL of plasma in an Eppendorf tube followed by vigorous vortex shaking for 5 min at room temperature and centrifugation at 4000 *g* for 10 min at 4 °C. The supernatant was carefully collected avoiding contamination with the precipitated proteins, transferred to another Eppendorf tube and the resulting supernatant was taken to dryness under nitrogen stream and stored to −80 °C until analysis. The pellet was homogenized again, with 200 μL of a mixture of (2:1) chloroform/methanol mixture, using a pellet mixer (2 min), to extract lipophilic metabolites and centrifuged (10,000 *g* at 4 °C for 10 min). Finally, the resulting supernatant was taken to dryness under nitrogen stream and stored to −80 °C until analysis.

The polar and lipophilic extracts were reconstituted to 200 μL of (1:1) chloroform/water mixture before the analysis by ESI-MS. For data acquisitions by positive ionization, 0.1% formic acid was added to polar extract and 30 mM of ammonium acetate to lipophilic extract. In the case of negative ionization intact extracts were directly infused to the mass spectrometer.

2.8. Metabolomic study of plasma of mice (M. musculus) under As/Cd exposure by GC–MS

Plasma was thawed at 4 °C and vortex-mixed before use. For the extraction of metabolites 100 μL of plasma were mixed with 400 μL of 1:1 methanol/ethanol mixture in an Eppendorf tube and vortexed for 5 min at room temperature, followed by centrifugation at 4000 *g* for 10 min at 4 °C. The supernatant was transferred to another Eppendorf tube and dried under nitrogen stream. All the dried samples were derivatized with 50 μL methoxylamine hydrochloride (20 mg mL^{-1} in pyridine) at 70 °C for 40 min, for protection of carbonyl groups by methoxymation, followed by treatment with 50 μL of MSTFA containing 1% of TMCS at 50 °C for 40 min, to derivatizate primary amines and primary and secondary hydroxy groups. TMCS participates in the derivatization of amides, secondary amines and hindered hydroxy groups. Finally, the derivatized samples were vortex-mixed for 2 min and centrifuged at 4000 *g* for 5 min to collect the supernatant for GC analysis.

Chromatography was performed on a Factor Four capillary column VF-5MS 30 m × 0.25 mm ID, with 0.25 μm of film thickness (Varian). The injector temperature was kept at 280 °C. Helium carrier gas was used at a constant flow rate of 1 mL/min. To acquire a good separation, the column temperature was initially maintained at 60 °C for 5 min, and then increased from 60 to 140 °C at a rate of 7 °C/min for 4 min. Then,

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the column temperature was increased to180 °C at 5°°C/min for another 6 min. After that, the temperature was increased to 280 °C at 5 °C/min, and held for 2 min. For mass spectrometry detection, ionization was carried out by electronic impact (EI) with a voltage of 70 eV, using full scan mode in the m/z range 35–650, with an ion source temperature of 200 °C. For analysis, 1 μl of sample was injected in splitless mode. The identification of endogenous metabolites was based on comparison with the corresponding standards according to their retention times and mass spectra characteristics by searching on NIST Mass Spectral Library (NIST 02).

2.9. Histopathological study of liver from mice under As/Cd exposure

Liver sample animals were excised as described above and immediately fixed in 4% neutral buffered formalin followed by dehydration in increasing grades of alcohol, clearing in xylene, and embedding in paraffin wax. Liver sections (4 mm thickness) obtained in a Leica Leitz 1512 precision rotary microtome (Leitz, Wetzlar, Germany) were stained with hematoxylin and eosin (H&E). The slides were blinded and analyzed by light microscopy for liver injury [18].

3. Results

3.1. Biochemical parameters in blood of mice under controlled exposure to As/Cd

Blood sampling work was performed by the same skilled technician for all samples, and all manipulations performed before and after blood collection were accurately settled, so that variability caused by blood sampling was negligible. Therefore, differences in the values assessed reflect factors directly associated with the blood sampling method, including handling stress, anesthesia, hemolysis, and tissue damage. In the present study, the level of hemolysis in all serum samples was scored by direct observation. The results obtained in the last day of the exposure experiment (12th day) are shown in Table 1.

3.2. Total metals distribution of arsenic and cadmium in mice M. musculus under both toxic metals exposure

The presence of arsenic and cadmium in the organs (liver and kidney) and plasma of *M. musculus* subjected to controlled exposure to As/Cd was evaluated by using ICP-ORS-MS, and the results are shown in Table 2. Recovery experiments were performed by spiking the extracts with 1, 5, 10 or 50 ng g^{-1} of analytes depending on the relative concentration of either one in the extracts. The results are also shown in Table 2 and confirm quantitative recoveries in all the cases. Instrumental detection limits are also given in this table.

The distribution of arsenic and cadmium in liver, kidney and plasma samples from mice exposed to As/Cd can be observed. An increased concentration of arsenic is obtained in mice plasma exposed to As in comparison with those exposed to As/Cd. Similar results are obtained for Cd concentrations in plasma. However, in kidney, the highest concentrations of As are obtained in mice exposed to As/Cd. In mammals, highly toxic inorganic arsenic is mainly metabolized in liver, after absorption from gastrointestinal tract, to produce methylated species such as MA^V and DMA^V, which are excreted by urine [19,20]. In this sense, our results show a major excretion of As when Cd is administered simultaneously (Table 2). In contrast, the major accumulation of Cd in kidney cytosolic extract is obtained when this element is administered isolated to mice. Since the most important interaction between these elements was observed in the liver, the cytosolic extract of this organ was used to study the biological response of exposed mice by SEC-ICP-ORS-MS.

3.3. Profiles of As and Cd-containing biomolecules in liver of M. musculus under As/Cd exposure by SEC-ICP-ORS-MS

To check the presence and potential interactions of metal-biomolecules in liver of *M. musculus* exposed to As/Cd the coupling SEC-ICP-MS was used, obtaining As and Cd-traced peaks from cytosolic fractions of liver (Fig. 1).

In Fig. 1 can be observed the presence of low molecular mass As species (<300 Da) in liver cytosolic extracts analyzed

Table 1 – Clinical parameters in blood from *Mus musculus* mice under As/Cd controlled exposure after twelve days of exposure.

Clinical parameters (mean ± SD)	Bilirubin mg/dL	Ferritine mg/dL	Albumin gr/dL	LDL mg/dL	HDL mg/dL	Alanine transpherase UI/L
CONTROL GROUP	0.07 ± 0.01	207 ± 9	3.4 ± 0.5	64 ± 5	99 ± 8	106 ± 8
As GROUP	0.07 ± 0.02	202 ± 11	3.6 ± 0.7	85 ± 6	108 ± 11	61 ± 11
Cd GROUP	0.05 ± 0.02	243 ± 15	3.3 ± 0.4	94 ± 6	115 ± 9	103 ± 9
As/Cd GROUP	0.02 ± 0.01	250 ± 17	3.5 ± 0.5	82 ± 4	107 ± 14	150 ± 15

Clinical parameters (mean ± SD)	Alkaline phosphatase UI/L	Amilase UI/L	Triglycerides mg/dL	Lipase UI/L	Creatinine mg/dL	Aspartate transpherase UI/L
CONTROL GROUP	149 ± 11	3515 ± 251	172 ± 14	32 ± 4	0.24 ± 0.04	384 ± 21
As GROUP	131 ± 13	3290 ± 303	203 ± 12	24 ± 3	0.36 ± 0.02	381 ± 32
Cd GROUP	157 ± 15	2989 ± 189	173 ± 14	35 ± 5	0.22 ± 0.02	719 ± 24
As/Cd GROUP	127 ± 12	3455 ± 225	241 ± 18	20 ± 4	0.25 ± 0.03	952 ± 62

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Table 2 – Quantification of arsenic and cadmium in plasma, liver and kidney of mice under As/Cd exposure by ICP-ORS-MS.

Tissue/Biological fluids	GROUP OF EXPOSURE	Total arsenic and cadmium concentrations (mean ± SD)								Recovery (%)	Limit of detection (LOD) (ng g^{-1})
		6th Day of exposure				12th Day of exposure					
		CONTROL GROUP	As GROUP	Cd GROUP	As/Cd GROUP	CONTROL GROUP	As GROUP	Cd GROUP	As/Cd GROUP		
Plasma	As (ng g^{-1})	38.2 ± 4.2	162 ± 9.1	32 ± 3.9	41.4 ± 5.6	32.1 ± 4.1	225 ± 18	25.3 ± 2.4	68.2 ± 4.9	102	0.012
	Cd (ng g^{-1})	4.71 ± 0.71	4.9 ± 0.4	59 ± 8.4	48.1 ± 6.7	5.56 ± 0.64	6.01 ± 0.32	91.1 ± 11	52.3 ± 6.2	99	0.024
Liver	As (ng g^{-1})	103 ± 8.9	516 ± 37	138 ± 12	602 ± 35	79.6 ± 9.2	750 ± 44	63.2 ± 4.7	717 ± 52	104	0.032
	Cd (ng g^{-1})	12.7 ± 2.5	14.8 ± 1.5	296 ± 7.5	152 ± 14	16.2 ± 3.1	18.5 ± 2.7	911 ± 9.2	121 ± 7.1	89	0.011
Kidneys	As (ng g^{-1})	48.4 ± 6.4	184 ± 26	102 ± 11	234 ± 21	59.4 ± 5.5	209 ± 16	109 ± 5.7	321 ± 22	98	0.033
	Cd (ng g^{-1})	1.22 ± 0.12	0.84 ± 0.11	37.2 ± 4.2	18.6 ± 3.4	1.12 ± 0.091	0.54 ± 0.081	44.4 ± 3.2	21.2 ± 2.6	101	0.021

by SEC-ICP-MS. The higher intensity of signals was obtained from liver of mice exposed to As/Cd during 6 days (Fig. 1A), in which As concentration is the highest (Table 2). In Fig. 1A and B a peak traced by As can be observed at about 32 kDa in the liver extract of exposed mice. This fact can be related to the interaction of As with enzymes such as carbonic anhydrase (CA) and superoxide dismutase (Cu/Zn-SOD) with molecular masses of 35 kDa and 32 kDa, respectively. The increase of this peak is more pronounced when As is administered alone in comparison with the joint administration As/Cd. Another peak traced by As was observed at about 70 kDa, which increases with the exposure during 6 days, this can be related to the well-known affinity of arsenite to albumin and hemoglobin, with molecular masses of 67 kDa and 68 kDa, respectively [21,22]. In this case, this peak presents higher intensity in mice exposed to the mixture As/Cd during 6 days (Fig. 2A).

On the other hand, higher intensity of signals from Cd associated to MT (7 kDa) is observed in liver cytosolic extracts from mice under Cd exposure (Fig. 2C and D). This peak is clearly more pronounced when cadmium is administered alone. An important depletion of this peak when As/Cd are simultaneously ingested is remarkable (Fig. 2C and D). In relation to this, the induction of Cd-metallothioneins in mice (*M. musculus*) exposed to high concentrations of Cd has been reported [3], and these experimental data confirm the antagonistic interactions among Cd, Zn, Cu, as well as the differential rate of excretion of these elements from kidney/liver under increasing exposure as a consequence of the major affinity of Cd for the thiol groups of MTs, which replaces copper and Zn due to its more electrophilic character [23]. The induction of Cd-MT is also a consequence of the role of MTs in Cd detoxification [24,25]. In addition, a second Cd peak at about 32 kDa can be observed, whose intensity increases with exposure to Cd (Fig. 2C and D). In this sense, it is well known that Cd presents the ability to replace Zn in several metalloproteins such as carbonic anhydrase enzyme (CA), resulting in a decrease in catalytic efficiency reported by other authors [26].

3.4. *Speciation of selenium in plasma of mice (M. musculus) under cadmium exposure by SEC-AF-HPLC-SUID-ICP-ORS-MS*

Quantification of Se containing proteins (selenoprotein P – SeP, extracellular glutathione peroxidase – eGPx and selenoalbumin – SeAlb) and low molecular weight Se species has been performed in mice plasma using the proposed speciation method. Selenium concentration in selenoproteins is in good accordance with total Se concentrations determined by IDA-ICP-ORS-MS after acid digestion (Table 3).

The effect of mice independent exposure to As or Cd on selenium containing proteins present in plasma is similar, decreasing the concentration of SeP, SeAlb and Se-metabolites and increasing the level of eGPx (Table 3). It has been documented that Cd and Se interact in the body of mammals, and the co-administration of both elements reduces the toxicity of each other [27]. Consequently, the Se level in plasma decreases under Cd exposure in rats subjected to oral administration [27]. This fact explains the decreased levels of SeP in mice plasma under Cd exposure (Table 3). However, the depletion of SeP concentration is reversed under As/Cd exposure. Since

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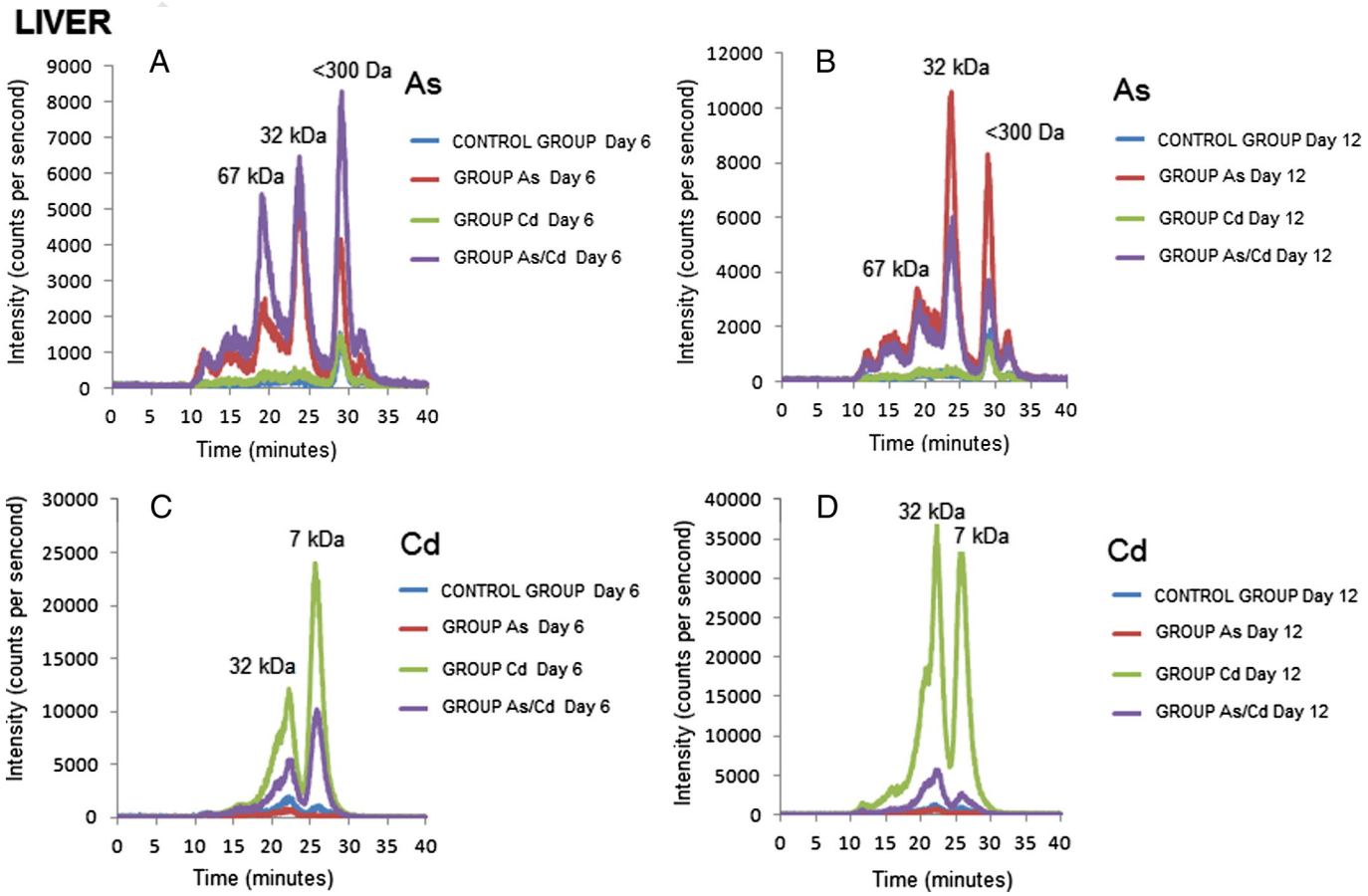

Fig. 1 – Up/down regulation of metal-biomolecule complexes in liver of *Mus musculus* exposed to arsenic/cadmium assessed by molecular mass distribution with SEC-ICP-MS. Chromatographic conditions: column, Superdex™-200 (10 × 300 × 13 μm); mobile phase, ammonium acetate 20 mM (pH 7.4); flow rate 0.7 ml min^{-1}; injection volume, 50 μL.

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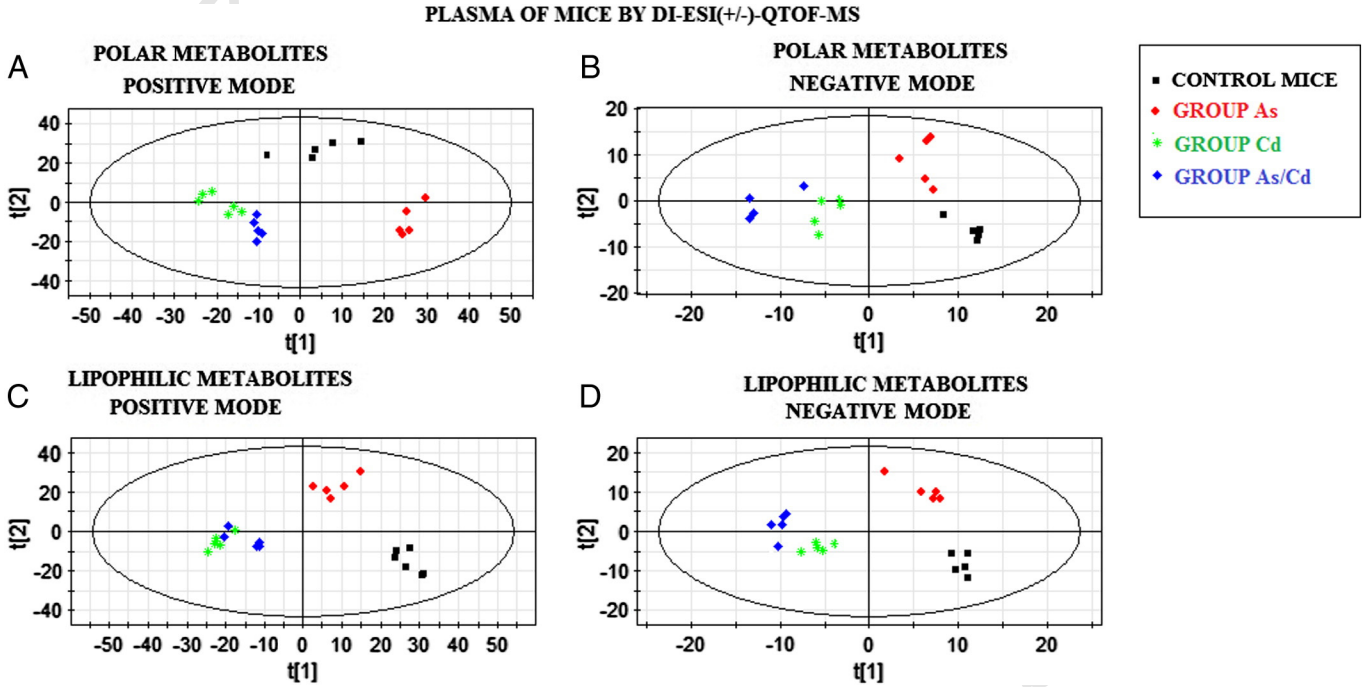

Fig. 2 – Score plots of PLS-DA for ESI+ and ESI– ionization modes of polar and lipophilic metabolites from mice plasma. Black squares: control group; red circles: mice exposed to As during 12 days; green asterisk: mice exposed to Cd during 12 days; blue diamonds: mice exposed to As/Cd mice during 12 days.

Table 3 – Quantification of selenium species in mice plasma (*Mus musculus*) under As/Cd exposure.

	Selenium containing proteins concentrations (mean ± SD)					
	12th Day of exposure					
GROUP OF EXPOSURE	*CONTROL GROUP*	*As GROUP*	*Cd GROUP*	*As/Cd GROUP*	BCR-637	Limits of detection (LOD)
GPx (ng g^{-1})	5.61 ± 0.42	5.92 ± 0.51	6.12 ± 0.84	7.41 ± 1.1	11 ± 2	0.21
SeP (ng g^{-1})	148 ± 5.2	132 ± 11	121 ± 9.2	165 ± 13	52 ± 3	0.72
SeAlb (ng g^{-1})	16.1 ± 0.82	12.1 ± 1.4	8.24 ± 0.91	8.8 ± 0.82	17 ± 2	1.3
Se-metabolites (ng g^{-1})	32.3 ± 6.2	3.24 ± 1.8	5.45 ± 2.1	<LOD	<LOD	0.24
Sum of species (ng g^{-1})	202 ± 5.2	153 ± 6.2	141 ± 5.1	181 ± 9.1	80 ± 3	–
Total selenium (ng g^{-1})	203 ± 3.1	151 ± 2.2	158 ± 4.6	182 ± 2.2	81 ± 1	0.036

Se-metabolites and SeAlb are required for the synthesis of selenoproteins in liver for later transport to plasma [28], Decreased levels of selenium metabolites in mice plasma after the administration of As/Cd, as well as a little reduction of SeAlb levels along the exposure can be observed in Table 3, which supports the hypothesis that SeAlb and selenometabolites have been transported to liver for the synthesis of required SeP. Finally, the increased levels of eGPx in plasma (Table 3) can be related with the transport of this enzyme from liver to plasma to neutralize lipid peroxidation [29] caused by the exposure to As/Cd.

3.5. *Metabolomic study of plasma from mice (M. musculus) under As/Cd exposure by DI-ESI(±)-QTOF-MS and GC–MS*

In order to discriminate between the groups of mice differentially exposed to As/Cd, a partial least squares discriminant analysis (PLS-DA) was performed employing the intensities of the m/z signals in the polar and lipophilic extracts from mice plasma, using positive and negative ionization mode of acquisition by DI-ESI-QTOF-MS. The models built with polar and lipophilic metabolites allow a good classification of samples in different groups, which are shown by the respective score plots (Fig. 2). To identify which variables were responsible for this separation, the *Variable Influence on the Projection* (VIP) parameter was used. VIP is a weighted sum of squares of the PLS-DA weight that indicates the importance of the variable to the whole model. Thus it is possible to select variables with the most significant contribution in discriminating between metabolomic profiles corresponding to groups of exposure against controls. Only metabolites with VIP > 2 have been considered good biomarkers of As/Cd exposure. The values of R^2Y (cum) and Q^2 (cum) of the combined model are 0.90–0.99 and 0.80–0.95, respectively, indicating that a combination of datasets between groups provides the best classification and prediction. In addition, the complementarity of using both ionization modes for polar and lipophilic metabolites is remarkable (see Table 4).

In addition, GC–MS was applied as complementary metabolomic approach to confirm and quantify altered metabolites established by DI-ESI(±)-QTOF-MS. For this purpose, three derivatizing reagents were used for plasma samples in order to get as much metabolic information as possible. Plasma metabolic profiles of five samples of mice plasma from each group of exposure were obtained by GC–MS. The concentrations of metabolites are shown in Table 5.

These metabolic changes in *GROUP As/Cd* can be related to perturbations in different metabolic cycles, such as inhibition of tricarboxylic acid cycle (TCA) marked by decreasing of glucose and increasing concentration in lactic acid under Cd exposure. However, As exposure produces a decrease of lactic acid levels and As/Cd does not provoke any alteration in this metabolite (Table 5). Further perturbations of energy metabolism intermediates, such as citric acid, isocitric acid, α-ketoglutarate and glutamic acid were also observed (Tables 4 and 5). Under As exposure, increased concentrations of intermediate energy

Table 4 – Biomarkers from plasma of mice (*Mus musculus*) exposed to arsenic/cadmium during 12 days.

Metabolites	*m/z*	*Mode of acquisition*	*GROUP As*	*GROUP Cd*	*GROUP As/Cd*
Taurine	124.01 (H^-)	ESI(–)	↑	↓	↑
Choline	104.09 (H^+)	ESI(+)	↑	↑	↑
Glutamic acid	148.05 (H^+)	ESI(+)	↑	↓	↑
Citric acid	193.03 (H^+)	ESI(+)	↑	↓	↑
Glucose	203.05 (Na^+)	ESI(+)	↓	↓	↓↓
	215.03 (Cl^-)	ESI (–)	↓	↓	↓↓
Pipecolic acid	130.08 (H^+)	ESI(+)	↑	↑	↑
Arachidonic acid	303.24 (H^-)	ESI(–)	↑	↑	↑
Palmitic acid	255.42 (H^-)	ESI(–)	↑	↑	↑
Lyso-phosphatidylcholines (Lyso-PC)	450–600	ESI(+)/ESI(–)	↑	↑	↑
Phosphatidylcholines (PC)	700–850	ESI(+)	↓	↓	↓
Diglycerides	600–700	ESI(+)/ESI(–)	↑	↑	↑↑
Triglycerides	850–950	ESI(+)/ESI(–)	↑	↑	↑↑

Variations compared to control mice: ↑, increasing signal intensity; ↓, decreasing signal intensity.

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Table 5 – Quantification of mice plasma metabolites (*Mus musculus*) exposed to arsenic and cadmium by GC–MS.

Plasma metabolites (mmol L^{-1})	*Retention time*	*GRUPO CONTROL Día12 (n = 5)*	*GRUPO ARSÉNICO Día12 (n = 5)*	*GRUPO CADMIO Día12 (n = 5)*	*GRUPO ARSÉNICO/CADMIO Día12 (n = 5)*
	(min)	Media ± SD	Media ± SD	Media ± SD	Media ± SD
Lactic acid	5.6	**1.66 ± 0.11**	**1.09 ± 0.19**	**1.98 ± 0.28**	**1.83 ± 0.31**
Alanine	6.0	**0.326 ± 0.021**	**0.254 ± 0.032**	**0.284 ± 0.024**	**0.233 ± 0.028**
Valine	11.6	0.216 ± 0.042	0.222 ± 0.032	0.218 ± 0.023	0.231 ± 0.016
Urea	12.5	360 ± 12	377 ± 29	354 ± 15	348 ± 24
Isoleucine	13.2	0.0681 ± 0.009	0.0669 ± 0.015	0.0465 ± 0.009	0.0521 ± 0.007
Proline	13.3	0.142 ± 0.005	0.138 ± 0.012	0.151 ± 0.022	0.154 ± 0.016
Glycine	13.4	**0.184 ± 0.009**	**0.224 ± 0.018**	**0.218 ± 0.018**	**0.242 ± 0.011**
Serine	14.5	0.0982 ± 0.003	0.102 ± 0.01	0.111 ± 0.022	0.108 ± 0.009
Threonine	15.0	0.129 ± 0.008	0.131 ± 0.014	0.122 ± 0.014	0.128 ± 0.016
Aspartic acid	17.2	0.086 ± 0.009	0.089 ± 0.008	0.092 ± 0.012	0.087 ± 0.009
Glutamine	22.1	0.648 ± 0.021	0.651 ± 0.018	0.669 ± 0.031	0.653 ± 0.032
Glutamic acid	23.1	**0.134 ± 0.08**	**0.175 ± 0.21**	**0.109 ± 0.014**	**0.194 ± 0.016**
α-Ketoglutarate	23.5	**0.144 ± 0.007**	**0.204 ± 0.021**	**0.092 ± 0.009**	**0.153 ± 0.012**
Phenylalanine	25.5	0.0562 ± 0.002	0.0541 ± 0.003	0.0563 ± 0.006	0.0568 ± 0.004
Isocitric acid	25.9	**0.512 ± 0.021**	**0.589 ± 0.033**	**0.467 ± 0.025**	**0.534 ± 0.018**
Citric acid	27.2	**3.21 ± 0.24**	**3.84 ± 0.36**	**2.92 ± 0.17**	**3.52 ± 0.25**
Fructose	27.9	0.935 ± 0.033	0.942 ± 0.045	0.899 ± 0.031	0.912 ± 0.032
Galactose	29.0	0.125 ± 0.013	0.114 ± 0.021	0.116 ± 0.009	0.121 ± 0.011
Glucose	29.9	**8.24 ± 0.47**	**6.85 ± 0.89**	**6.26 ± 0.71**	**6.14 ± 0.54**
Tyrosine	31.0	0.115 ± 0.012	0.121 ± 0.015	0.111 ± 0.009	0.109 ± 0.011
Uric acid	37.0	0.142 ± 0.006	0.192 ± 0.021	0.135 ± 0.011	0.138 ± 0.009
Tryptophan	38.1	0.094 ± 0.011	0.101 ± 0.01	0.106 ± 0.009	0.098 ± 0.010

metabolites were obtained (Tables 4 and 5). This fact has been previously reported in *M. musculus* mice exposed to arsenic [4] and has been related with the synthesis of glutathione (GSH) [4]. Under As/Cd exposure, similar effects were observed.

Perturbations of amino acid concentrations related to toxic exposure [30] can also be seen in Table 4, which shows decreasing levels of taurine and alanine.

The degradation of phospholipids such as phosphatidyl-choline (PC) and the correlative release of fatty acid from the PC moiety, generating a series of products including lysophosphatidyl-choline (LPC), glycerol-phosphocholine, and finally phosphocholine and choline whose concentration increases with As/Cd exposure is very remarkable (Table 4). This process induces degradation of membrane phospholipids and cell apoptosis. Consequently, the levels of free fatty acid increase, which is confirmed by ESI(+) and ESI(–) analysis of liver lipophilic extract, which increases the presence of PUFAs (Table 4).

Finally, triglycerides and diglycerides levels are also altered under the action of arsenic/cadmium, increasing the levels under exposure. The mass spectra show several diglycerides and triglycerides from lipophilic extracts in the m/z ranges of 600–700 and 850–950, respectively (data no shown). These results are in agreement with previous works from Griffin et al. in bank voles under arsenic exposure [31] and rats under cadmium exposure [32]. Increased biosynthesis of triglycerides and diacylglycerol has also been found in apoptotic KB cells [33]. However, the mechanism for the relationship between increased lipids metabolites and cell apoptosis still remains unclear. Accumulation of lipid might well be associated with arsenic and cadmium induced cell apoptosis. The accumulation of DGs and TGs is more pronounced when As and Cd are administered at the same time (Table 4), which is confirmed by the results of TGs in Table 1.

3.6. Histopathological study of liver from mice subjected to As/Cd exposure

The pathological changes in response to As_2O_3 and $CdCl_2$ exposure were examined and compared among different experimental groups in the liver. The liver is a primary defense organ that detoxifies drugs and xenobiotics, which increase the probability to injury in this organ. Normal morphology of liver histological sections from mice *CONTROL GROUP* is shown in Fig. 4. Arsenic exposure originates important hepatic damage, such as steatosis, inflammation, significant fibrosis in periportal areas and necrosis (Fig. 4, *As GROUP*). Cadmium administration resulted in sinusoidal congestion, Mallory bodies' appearance and multifocal hepatic necrosis after 12 days of exposure (Fig. 4, *Cd GROUP*). Finally, joint exposure to cadmium and arsenic causes a more severe hepatotoxicity (pyknotic nuclei, karyolysis, infiltration of blood cells into the Disse's space) and multifocal necrosis that result in major liver injuries, related to loss of architecture and vacuolization, with a more extensive tissue congestion (Fig. 4, *As/Cd GROUP*).

4. Discussion

Experiences in living organisms conducting exposure to multiple toxics, as is the case of As and Cd, reveal the interest of this kind of studies due to the interactions occurring between them along the complex biological processes, from toxic exposure to excretion and their toxicological consequences.

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These experiments reflect better what happen in contamination episodes, in which the simultaneous presence of several xenobiotics is frequent. It is also convenient to perform these toxicological studies involving several organs of the test-animal, since it allows a more comprehensive interpretation of metabolic processes that underlie the defense mechanism against toxic substances, this is the case of liver, kidney and plasma considered in the present study.

The exposure of mice *M. musculus* to As, Cd and As + Cd along 12 days confirms the occurrence of important interactions between these elements that reduce the presence of As in plasma when Cd is administrated simultaneously. Arsenic is metabolized in liver to methylated species to be excreted by urine and this process is enhanced by the presence of Cd, for this reason the presence of As in kidney is clearly higher (Table 2). Methylation of As in liver induced by the presence of Cd is confirmed by the marked increase of the As-peak related to low molecular mass arsenic species (<300 Da) in Fig. 1A after 6 days of exposure to As + Cd. The interaction of Cd with As is also confirmed by the intensity of Cd-peak at 7 kDa, associated to Cd-MT the form of excretion of this toxic element, which is higher in the case of Cd exposure but clearly decreases when As/Cd are simultaneously ingested (Fig. 1C and D).

On the other hand, blood is the more important transport fluid between liver and kidney involving carrier proteins that contribute to detoxification of As and Cd, specially selenoprotein P (SeP) and selenoalbumin (SeAlb). The SeP is the unique selenoprotein that contains several selenocysteine (SeCys) and cysteine (Cys) residues, which increase its availability to transport Se that can interacts with Cd for excretion. This fact explains the decreased levels of SeP in mice plasma under Cd exposure (Table 3). However, this reduction of SeP concentration is compensated under As/Cd exposure. In addition, extracellular glutathione peroxidase (eGPx), an antioxidant enzyme that reduces the presence of lipid hydroperoxides in plasma [29], increase with the exposure to Cd, but specially to As/Cd (Table 3) due the neutralization of lipid peroxidation caused by the mixtures of these toxic metals.

These results based on metallomic approaches can be combined with metabolomic procedures to get a more comprehensive assessment of harmful effects of toxic elements exposure, and the biological response elicited in living organisms as mice. In this sense, Cd or As exposure triggers inhibition of tricarboxylic acid cycle but As/Cd exposure does not alter this cycle and changes in lactic acid concentration was not observed (Table 5). Aminoaciduria caused by Cd exposure is associated to decreasing energy production within renal mitochondria, which is a consequence of energetically limited re-uptake of involved metabolites in the glomerulus and proximal tubule. This fact possibly culminates in cellular necrosis or apoptosis [34].

On the other hand, As/Cd ingestion causes degradation of phospholipids, such as phosphatidylcholine, induced by phospholipase A2, and correlative increase of lysophosphatidylcholine, glycerol–phosphocholine, and finally phosphocholine and choline. In addition, free fatty acid released from PC also increases with As/Cd exposure (Table 4). These processes lead to cell membrane degradation and apoptosis that is also related with the increase of triglycerides and diglycerides levels provoked by these toxics.

Finally, the effect of As/Cd exposure on mice organ histopathology is another valuable point to deep insight into the consequences of these elements on mammals exposed. It has been described that the arsenic administered together with cadmium damaged the liver [35], kidney [36,37] and bladder [38], however they do not seem to affect either the lung or testis [38,39]. In the present study, blood chemistry clearly shows toxic cirrhosis induced by Cd, which is aggravated with the joint exposure to As (see also Fig. 3). A cirrhotic liver leads to increased transaminases (alanine transferase and aspartate transferase) which injure the hepatocytes. On the other hand, toxicity causes reduction of bilirubin and albumin, as there is less ability to metabolize hepatic heme and synthesize albumin. In *As/Cd GROUP* these effects produce more severe liver injury, due to the additional appearance of hemorrhage accompanied by severe cirrhosis, and irreversible condensation of chromatin in the nucleus of cells (pyknosis) undergoing necrosis or apoptosis (Fig. 3, *As/Cd GROUP*, B). The Fig. 3, *Cd GROUP*, B, shows a detail of necrotic tissue with abundance of material in the form of lump eosinophils (Mallory bodies) surrounded by neutrophils.

The increase of ferritin observed in Fig. 3. *As GROUP*, B; *Cd GROUP*, A and B; and *As/Cd GROUP*, A, reflects inflammatory processes, and that ferritin is an acute phase reactant protein that is elevated in all hepatocellular swelling. This process is more marked in *As/Cd GROUP*, suggesting that co-exposure to Cd and inorganic arsenic gives rise to more pronounced hepatic damage than exposure to each of the elements separately.

Histologically, *Cd GROUP* presents also thickening of sinusoids, focused infiltration of red blood cells and accumulation of lipofuscin pigment (Fig. 3, *Cd GROUP*, A), classically attributed to aging and decreased metabolic activity [40,41], but more recently it has been related with portal lymphadenopathy [42] and oxidative stress. Both are closely connected to disturbances of proteostasis by protein oxidation and impairment of proteasomal system. The final consequence is the accumulation of highly cross-linked undegradable aggregates such as lipofuscin, which can be considered as the long-term result of a decreased degradation of oxidized proteins and increase of intracellular free radical formation. These aggregates of damaged proteins are detrimental to normal cell functions [43].

In the *As GROUP* increase of transaminases or decrease of bilirubin in comparison to *CONTROL GROUP* are not observed, suggesting the absence of liver involvement. Therefore, we have to build on the histological studies to determine the degree of cell injury. At low magnification we can see the appearance of perivenular congestion (Fig. 3, *As GROUP*, A), accompanied by pathological steatosis and cirrhosis (Fig. 3, *As GROUP*, B). It is well known that perturbations in lyso-phosphatidylcholines levels are associated to this pathology [44].

5. Conclusion

This work illustrated the potential of combined use of a metabolomic approach, based on organic mass spectrometry for the study of biochemical effects induced by As/Cd exposure, with a metallomic approach, based on inorganic mass spectrometry for metals/metalloids-biomolecules and metabolites characterization in mice exposed to both elements. Interactions

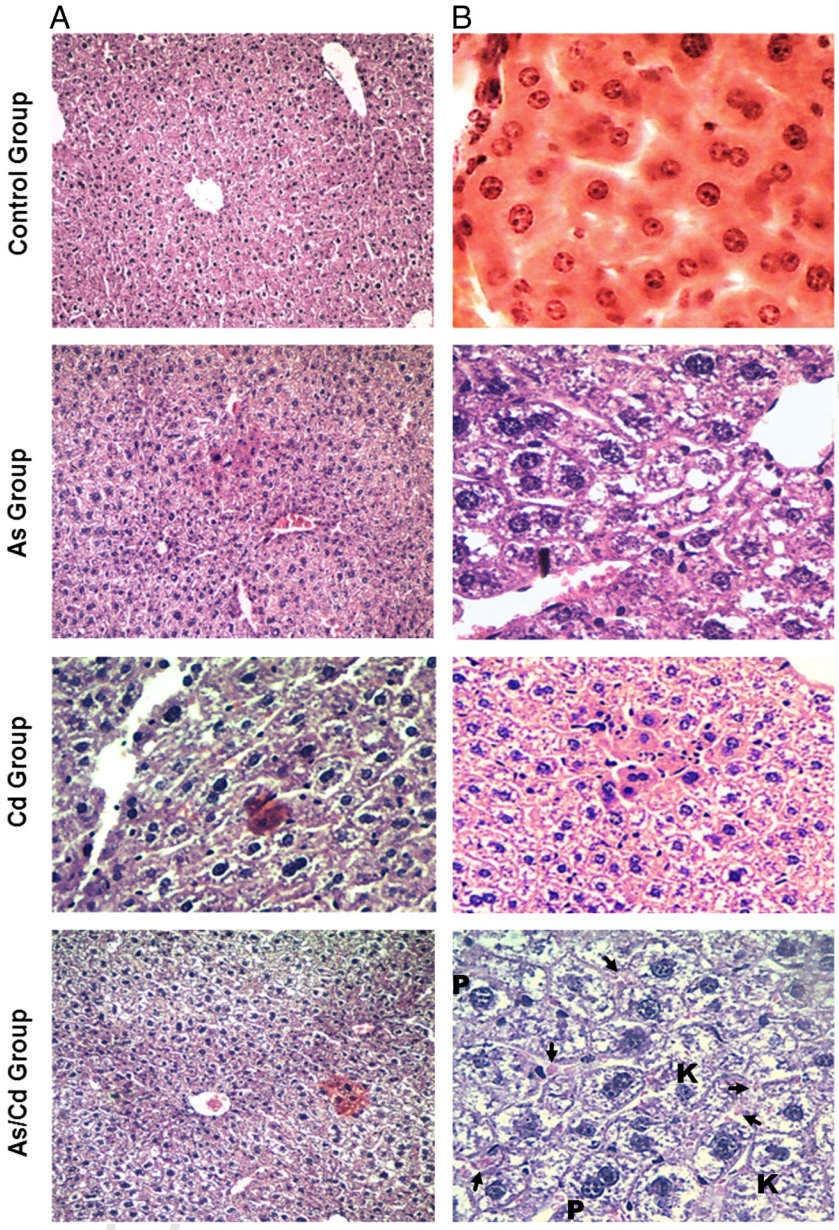

Fig. 3 – Histophatological study of liver from mice (*Mus musculus*) under metal exposure during 12 days. Liver sections were prepared as described under Materials and Methods and stained with H&E to assess liver pathologic characteristics. The magnification of the sections is 100-fold (*CONTROL GROUP*, remaining sections. (P), pyknotic nuclei, (K), karyolysis. Arrows show widespread infiltration of red blood cells in the space of Disse.

in the distribution and accumulation of arsenic and cadmium were obtained when both toxic metals are administered together. In addition, antagonistic interactions with selenium containing proteins (mainly SeP) in the bloodstream have been observed when both xenobiotics are ingested at the same time. Finally, important interactions in essential metabolic pathways, such a breakdown of membrane phospholipids, more pronounced under As/Cd exposure, was obtained. These effects are corroborated with histopathological evaluations of liver injury and complemented with the measurement of biochemical parameters in blood. Administration of heavy metal and metalloid, together or separately, resulted in differential liver injury, which has been characterized by the predominance of steatosis (non-alcoholic fatty liver disease, NAFLD) and infiltration of blood cells into the space of Disse. Therefore, the complementary application with metallomics and metabolomics approaches has shown to be a valuable experimental approximation to get overall information and conclusions in relation to toxicological studies.

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REFERENCES

[1] García-Barrera T, Gómez-Ariza JL, González-Fernández M, Moreno F, García-Sevillano MA, Gómez-Jacinto V. Biological responses related to agonistic, antagonistic and synergistic interactions of chemical species. Anal Bioanal Chem 2012;403:2237-225.

[2] García-Sevillano MA, Jara-Biedma R, González-Fernández M, García-Barrera T, Gómez-Ariza JL. Metal interactions in mice under environmental stress. Biometals 2013;26:651–66.

[3] Jara-Biedma R, González-Dominguez R, García-Barrera T, López-Barea J, Pueyo C, Gómez-Ariza JL. Evolution of metallotionein isoforms complexes in hepatic cells of *Mus musculus* along cadmium exposure. Biometals 2013;26:639–50.

[4] García-Sevillano MA, García-Barrera T, Navarro F, Gómez-Ariza JL. Analysis of the biological response of mouse liver (*Mus musculus*) exposed to As_2O_3 based on integrated -*omics* approaches. Metallomics 2013;5:1644–55.

[5] González-Fernández M, García-Sevillano MA, Jara-Biedma R, García-Barrera T, Vioque A, López Barea J, et al. Size characterization of metal species in liver and brain from free-living (*Mus spretus*) and laboratory (*Mus musculus*) mice by SEC-ICP-MS: application to environmental contamination assessment. J Anal At Spectrom 2011;26:141–9.

[6] Dudley RE, Gammal LM, Klaassen CD. Cadmium-induced hepatic and renal injury in chronically exposed rats: likely role of hepatic cadmium–metallothionein in nephrotoxicity. Toxicol Appl Pharmacol 1985;77:414–26.

[7] Yáñez L, Carrizales L, Zanatta MT, Mejia JJ, Batres L, Diaz-Barriga F. Arsenic–cadmium interaction in rats. Toxicology 1991;67:227–34.

[8] Sanz-Medel A. Heteroatom(isotope)-tagged genomics and proteomics. Anal Bioanal Chem 2008;390:1–2.

[9] Bettmer J, Montes Bayón M, Ruiz Encinar J, Fernández Sánchez ML, Fernández de la Campa MR, Sanz Medel A. The emerging role of ICP-MS in proteomic analysis. J Proteomics 2009;72:989–1005.

[10] Gomez-Ariza JL, Garcia-Barrera T, Lorenzo F, Bernal V, Villegas MJ, Oliveira V. Use of mass spectrometry techniques for the characterization of metal bound to proteins (metallomics) in biological systems. Anal Chim Acta 2004;524:15–22.

[11] García-Sevillano MA, González-Fernández M, Jara-Biedma R, García-Barrera T, López-Barea J, Pueyo C, et al. Biological response of free-living mouse *Mus spretus* from Doñana National Park under environmental stress based on assessment of metal-binding biomolecules by SEC-ICP-MS. Anal Bioanal Chem 2012;404:1967–81.

[12] Bowen BM, Northen TR. Dealing with the unknown: metabolomics and metabolite atlases. J Am Soc Mass Spectrom 2010;21:1741–6.

[13] Goldsmith P, Fenton H, Morris-Stiff G, Ahmad N, Fisher J, Prasad KR. Metabonomics: a useful tool for the future surgeon. J Surg Res 2010;160:122–32.

[14] Dettmer K, Aronov AP, Hammock BD. Mass spectrometry-based metabolomics. Mass Spectrom Rev 2007;26:51–78.

[15] García-Sevillano MA, García-Barrera T, Gómez-Ariza JL. Development of a new column switching method for simultaneous speciation of selenometabolites and selenoproteins in human serum. J Chromatogr A 2013;1318:171–9.

[16] Sariego-Muñíz C, Marchante-Gayón JM, García-Alonso JI, Sanz Medel A. Speciation of essential elements in human serum using anion-exchange chromatography coupled to post-column isotope dilution analysis with double focusing ICP-MS. J Anal At Spectrom 2001;16:587–92.

[17] Vanhaecke F, Wannemacker G, Moens L, Dams R, Latcoczy C, Prohaska T, et al. Dependence of detector dead time on analyte mass number in inductively coupled plasma mass spectrometry. J Anal At Spectrom 1998;13:567–71.

[18] Drury RAV, Wallington EA. Carlton's histological techniques. 5th ed. New York: Oxford University Press; 1980.

[19] Del Razo LM, García-Vargas GG, Vargas H, Albores A, Gonsebatt ME, Montero R, et al. Altered profile of urinary arsenic metabolites in adults with chronic arsenicism. A pilot study. Arch Toxicol 1998;71:211–7.

[20] Hughes MF, Kenyon EM, Edwards BC, Mitchell CT, Del Razo LM, Thomas DJ. Accumulation and metabolism of arsenic in mice after repeated oral administration of arsenate. Toxicol Appl Pharmacol 2003;191:202–10.

[21] Bogdan GM, Samgayo-Reyest A, Aposhian HV. Arsenic binding proteins of mammalian systems: I. Isolation of three arsenite-binding proteins of rabbit liver. Toxicol Appl Pharmacol 2003;93:175–93.

[22] Lu M, Hailin W, Xing-Fang L, Arnold LL, Cohen SM, Le XC. Binding of dimethylarsinous acid to cys-13alpha of rat hemoglobin is responsible for the retention of arsenic in rat blood. Chem Res Toxicol 2007;20:27–37.

[23] Sasakura C, Suzuki KT. Biological interaction between transition metals (Ag, Cd and Hg), selenide/sulfide and selenoprotein P. J Inorg Biochem 1998;71:159–62.

[24] Shimoda R, Achanzar WE, Qu W, Nagamine T, Takagi H, Mori M, et al. Metallothionein is a potential negative regular of apoptosis. Toxicol Sci 2003;73:294–300.

[25] Yang MS, Lai KP, Cheng KY, Wong CKC. Changes in endogenous Zn and Cu distribution in different cytosolic protein fractions in mouse liver after administration of a single sublethal dose of CdCl2. Toxicology 2000;154:103–11.

[26] Xu Y, Feng L, Jeffrey PD, Shi Y, Morel FM. Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. Nature 2008;452:56-U3.

[27] Said L, Banni M, Kerkeni A, Said K, Messaoudi I. Influence of combined treatment with zinc and selenium on cadmium induced testicular pathophysiology in rat. Food Chem Toxicol 2010;48:2759–65.

[28] Papp LV, Lu J, Holmgren A, Khanna KK. From selenium to selenoproteins: synthesis, identity, and their role in human health. Antioxid Redox Signal 2007;9:775–806.

[29] Jones OAH, Walker LA, Nicholson JK, Shore RF, Griffin JL. Cellular acidosis in rodents exposed to cadmium is caused by adaptation of the tissue rather than an early effect of toxicity. Comp Biochem Physiol Part D Genomics Proteomics 2007;2:316–21.

[30] Connor SC, Wu W, Sweatman BC, Manini J, Haselden JN, Crowther DJ, et al. Effects of feeding and body weight loss on the ^{1}H NMR-based urine metabolic profiles of male Wistar Han rats: implications for biomarker discovery. Biomarkers 2004;9:156–79.

[31] Griffin JL, Walker LA, Shore RF, Nicholson JK. High-resolution magic angle spinning ^{1}H NMR spectroscopy studies on the renal biochemistry in the bank vole (*Clethrionomys glareolus*) and the effects of arsenic (As3+) toxicity. Xenobiotica 2001;31:377–85.

[32] Griffin JL, Walker LA, Shore RF, Nicholson JK. Metabolic profiling of chronic cadmium exposure in the rat. Chem Res Toxicol 2001;14:1428–34.

[33] Engelmann J, Henke J, Willker W, Kutscher B, Nossner G, Engel J, et al. Early stage monitoring of miltefosine induced

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apoptosis in KB cells by multinuclear NMR spectroscopy. Anticancer Res 1996;16:1429–39.
[34] Nicholson JK, Kendall MD, Osborn D. Cadmium and mercury nephrotoxicity. Nature 1983;304:633–5.
[35] Hochadel JF, Waalkes MP. Sequence of exposure to cadmium and arsenic determines the extent of toxic effects in male Fisher rats. Toxicology 1997;116:89–98.
[36] Nordberg GF, Jina T, Hong F, Zhang A, Buchet JP, Bernard A. Biomarkers of cadmium and arsenic interactions. Toxicol Appl Pharmacol 2005;206:191–7.
[37] Liu J, Liu Y, Habebu SM, Waalkes MP, Klaassen CD. Chronic combined exposure to cadmium and arsenic exacerbates nephrotoxicity, particularly in metallothionein-I/II null mice. Toxicology 2000;147:157–66.
[38] Jiang T, Huang Z, Chan JY, Zhang DD. Nrf2 protects against As(III)-induced damage in mouse liver and bladder. Toxicol Appl Pharmacol 2009;240:8–14.
[39] Hochadel JF, Waalkes MP. Sequence of exposure to cadmium and arsenic determines the extent of toxic effects in male Fischer rats. Toxicology 1997;116:89–98.
[40] Tygstrup N, Schiϕdt T, Winkler K. Correlation between pathological and clinical findings in 195 consecutive liver biopsies showing brown pigment. Gut 1965;6:194–9.
[41] Tauchi H, Hananouchi M. Accumulation of lipofuscin pigment in human hepatic cells from different races and in different environmental conditions. Mech Ageing Dev 1980;12:183–95.
[42] Hubscher SG, Harrison RF. Portal lymphadenopathy associated with lipofuscin in chronic cholestatic liver disease. J Clin Pathol 1989;42:1160–5.
[43] Höhn A, Grune T. Lipofuscin: formation, effects and role of macroautophagy. Redox Biol 2013;1:140–4.
[44] Puri P, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min HK, et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. Hepatology 2009;50:1827–38.

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