Effects of oral exposure to arsenite on arsenic metabolism and transport in rat kidney

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Abstract

Nephrotoxicity is within the recognized toxic effects of arsenic. In this study we assessed the effect of arsenite on the renal capacity to metabolize and handle arsenicals in rats exposed to drinking water with 0, 1, 5, or 10 ppm sodium arsenite for ten days. Arsenite treatment did not affect the gene expression of the main enzyme catalyzing methylation of arsenite, *As3mt*, while it reduced the expression of GSTO1 mRNA and protein. Arsenite decreased the expression of *Aqp3, Mrp1, Mrp4,* and *Mdr1b* (i.e., transporters and channels used by arsenic), but not that of *Aqp7, Glut1, Mrp2,* and *Mdr1a*. The protein abundance of AQP3 was also reduced by arsenite. Arsenite increased urinary NGAL and FABP3 and decreased Klotho plasma levels, without alteration of creatinine, which evidenced early tubular damage. Renal Klotho mRNA and protein expressions were also downregulated, which may exacerbate renal damage. No effect was observed in selected miRNAs putatively associated with renal injury. Plasma PTH and FGF23 were similar between groups, but arsenite decreased the renal expression of *Fgfr1* mRNA. In conclusion, exposure to arsenite alters the gene expression of proteins involved in the cellular handling of arsenical species and elicits tubular damage.

Keywords: arsenic, multidrug resistance proteins, aquaporins, renal damage.

1. Introduction

Chronic exposure to high levels of arsenic due to contaminated water or food sources is a human health problem affecting millions of people in several parts of the world (Nordstrom, 2002; World Bank, 2005). Inorganic arsenic – arsenate (AsV) and arsenite (AsIII) – is recognized as a multi-target carcinogen (especially in the skin, bladder and lungs) (Smith and Steinmaus, 2009, IARC, 2012), and although strong evidence is limited, it has also been associated with a wide range of adverse health effects, such as cardiovascular disease (Balakumar and Kaur, 2009; States et al., 2009), diabetes (Díaz-Villaseñor et al., 2007; Smith, 2013), reproductive disorders (Pizent et al., 2012; Sengupta et al., 2015), neurological disorders (Tyler and Allan 2014), and renal toxicity (Fowler, 2005).

Several mechanisms of arsenic toxicity have been described, such as interference with phosphate and sulfur metabolism, oxidative stress, genotoxicity, interference with signal transduction, stimulation of cellular proliferation, and alteration of DNA methylation (Hughes et al., 2011; Thomas, 2007). However, the severity of the effects depends on the ability of the different cells to manage the incoming concentrations of arsenic, and in turn this depends on the rates of uptake, metabolism, and efflux from a cell. Most inorganic arsenic enters a cell in the form of AsIII, given that AsV is rapidly reduced in the blood to arsenite, at least partially, (Villa-Bellosta and Sorribas, 2008). AsIII uptake pathways, in turn, include absorption through the channel proteins, aquaglyceroporins (AQP3, 7, 9, and 10), and the equilibrative D-glucose transporter (GLUT1) (Mukhopadhyay et al., 2014; Thomas, 2007). In the intestine, however, AsV seems to be absorbed through the sodium Pi cotransporter NaPi-IIb (SLC34A2) (Villa-Bellosta and Sorribas, 2010). Arsenicals then undergo extensive biotransformation through conversion into methylated metabolites and through glutathione-driven reduction. The trivalent oxidation state forms of arsenic (arsenite, mono-, and di-methylarsonous acid) are methylated primarily by arsenite methyltransferase (AS3MT,

Cyt19), a highly conserved enzyme that transfers the methyl group of S-adenosylmethionine to the arsenical molecule (Lin et al., 2002; Thomas et al., 2007). The resulting methylated pentavalent arsenicals (monomethylarsonic or dimethylarsinic acid) are then reduced to trivalent forms in the presence of glutathione, mainly by the activity of glutathione S-Transferase Omega 1 (GSTO1) (Zakharyan et al., 2001). The efflux of methylated and/or glutathione-conjugated arsenicals out of a cell relies mainly on the activity of members of two subfamilies of ATP-binding cassette transporters: ABCB (multidrug resistance proteins, MDR) and ABCC (multidrug resistance-associated proteins, MRP). Specifically, the transporters with demonstrated activity in cellular arsenical elimination include ABCB1/MDR1/P-glycoprotein (although it does not translocate glutathione-conjugates), ABCC1/MRP1, ABCC2/MRP2, and ABCC4/MRP4 (Banerjee et al., 2014; Leslie, 2012).

Arsenic is also a recognized nephrotoxic metalloid that triggers diverse events inside cells (e.g., intracellular glutathione depletion, activation of the caspase-3 and -9 pathways, p53-mediated apoptosis), which will ultimately lead to tubular injury (Robles-Osorio et al., 2015; Zheng et al., 2014). Long-term arsenic exposure has been recently associated with the development of a fibrotic phenotype in human kidney cells, probably mediated by DNA methylation (Chang and Singh, 2019). In rats treated with arsenate or dimethylarsinic acid, the highest concentrations of dimethylated arsenic were found in the blood, and similar values were observed in the kidney, liver, and lungs (Adair et al., 2007), thereby supporting the notion that the kidney is the primary target organ of arsenic toxicity.

The present work focusses on the effects arsenic exposure in the kidney. The aim of this study was to assess if arsenic exposure in rats altered the expression of proteins involved in the uptake, metabolism, and efflux of arsenicals in renal cells and if these changes could be related, at least in part, to nephrotoxicity. We found evidence of alteration of the gene expression of proteins involved in the cellular handling of arsenical species, as well as evidence of the early biomarkers of kidney damage.

Given the pivotal role of the kidneys in the homeostasis of inorganic phosphate and in the interactions of arsenic at different levels, we also investigated the effects of oral arsenite on the renal handling of Pi.

2. Materials and methods

2.1. Animal experimentation

All procedures were in accordance with European and Spanish legislation and were approved by the Ethical Committee for Animal Experiments of the University of Zaragoza.

Two-month old male Wistar rats (Janvier Labs, Saint Berthevin Cedex, France) were exposed to tap water with 0, 1, 5, or 10 ppm $NaAsO_2$ (sodium arsenite) for 10 days (4 animals per group). This experiment was performed twice with identical results, and therefore only one is shown. The doses were chosen considering that rats are less susceptible to arsenical toxicity than humans and other animal species (ATSDR, 2007; NRC, 1977). To estimate the actual arsenic intake, water consumption was measured every three days, and the rats were weighed at the beginning and end of the experiment. The concentration of arsenic in the tap (municipal) water is below the analytical detection level and much lower than the 10 ppb recommended limit. Animals were also fed a synthetic, arsenic-free food containing 0.6% inorganic phosphate (SAFE Diets, Augy, France) used in previous studies (e.g. Hortells et al., 2017).
On day 9, urine was collected for 24 h (with normal food and water treatments), after which the rats were anesthetized (day 10) taking one animal from each group in a rotating manner to avoid diurnal variations. Heparinized blood samples were collected from the abdominal aorta in order to measure blood gases and to obtain plasma by centrifugation. After being sacrificed, ice-cold renal medulla and the cortex were separated by quick manual dissection, frozen with liquid nitrogen, and stored at -80º C

until analyzed. In this work only kidney cortex sections were used because it expresses all known transporters for the different arsenical species.

2.2. Plasma and urine determinations

Blood gases and electrolytes (Na^+, K^+, Cl^-, pH, PCO_2, PO_2, tHb, SO_2, tCO_2, HCO_3^-, base excess, anion gap) were measured in a VetStat® system (IDEXX Laboratories, Westbrook, ME, USA). Plasma and urinary creatinine, Pi, and urea were determined by colorimetric kits (BioAssay Systems, Hayward, CA, USA), and total urinary protein was measured by a BCA Protein Assay reagent. Enzyme-linked immunosorbent assays were performed to determine urinary neutrophil gelatinase-associated lipocalin (NGAL) and fatty acid-binding protein 3 (FABP3, both from Elabscience Biotechnology, Wuhan, China), in addition to plasma intact parathyroid hormone (PTH, Immutopics, San Clemente, CA, USA), Klotho (BlueGene, Shanghai, China), and fibroblast growth factor 23 (FGF23, Kainos Laboratories, Tokyo, Japan).

2.3. mRNA and miRNA expression analysis

Total RNA was isolated from renal cortices using the Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), and complementary DNA was prepared from 1 µg of total RNA using the PrimeScript RT Reagent kit (Takara Clontech, Mountain View, CA, USA). All qPCR reactions were performed using SYBR Premix Ex Taq II (Takara) on a LightCycler 1.5 (Roche Applied Science, Mannheim, Germany). The primer sequences are presented in Table 1. For relative quantification, gene expression was normalized to both an endogenous control (acidic ribosomal phosphoprotein P0, *Arbp*) and a calibrator (pool of RNA from various samples).

Gene	Accession no.	Sense	Antisense	Range
As3mt	NM_080890.1	TTCGCCTCTTCAAACTCCC	TCCTCATCCACTTCAACAGC	809-953
Gsto1	NM_01007602	AGCCCGAGTGGTTCTTTGAG	TGCTTCATCCAGGTACTCGC	258-370
Aqp3	NM_031703.1	AGGCAGAGAATGTGAAGCTG	AGTCAGTGGACACTCAAGAGA	906-1014
Aqp7	NM_019157.2	CACCCACAATTCCACAATGG	ATATGAGCCACGGAACCAAG	246-386
Glut1	NM_138827.1	TGATTGGTTCCTTCTCTGTGG	CCCAGGATCAGCATCTCAAAG	438-582
Mrp1	NM_022281.2	TCATCATCCTACTGGCTACGC	CGAGAGGAGGCCACATAGAA	3359-3455
Mrp2	NM_012833.2	GCTGGATCTGGTACTGAAAGG	TGAAGAGGCAGTTTGTGAGG	4137-4246
Mrp4	NM_133411.1	GTATGGTGCTGTCCGGTTGA	TCTTGATCCTCCGAACGCTG	1071-1162
Mdr1a	NM_133401.1	GTTTCGCTATGCAGGTTGGC	AAGTGGGAGCGCAATTCCAT	199-283
Mdr1b	NM_012623.2	TGCTGTTGGCATATTCGGGA	CGTGGATGATAGCAGCGAGA	125-213
Fgfr1	NM_024146.1	CGTGGAGTTCATGTGCAAGG	TACGGCAAGTTGTCTGGACC	816-920
Klotho	NM_031336.1	TTTAGGGCGGCTCAGAAAGG	TCTCCGCCACTTCTTTGTCC	2176-2275
NaPi-IIa	NM_013030.1	CTCACTGGTGTTTGGCATTTC	AACAAGCACCACGAAGGC	1628-1721
Arbp	NM_022402.2	CACCTTCCCACTGGCTGAA	TCCTCCGACTCTTCCTTTGC	844-971

Table 1. Primer sequences used for mRNA expression analysis by real time PCR. *As3mt*, arsenite methyltransferase; *Gsto1, glutathione S-transferase omega 1; Aqp3 and 7*, aquaporin 3 and 7; *Glut1,* glucose transporter 1; *Mrp1, 2, and 4, multidrug resistant proteins 1, 2, and 4*; *Mdr1,* multidrug resistant protein/P-glycoprotein; *Fgfr1*, fibroblast growth factor receptor 1; *NaPi-IIa,* sodium-dependent phosphate transporter type II isoform a*; Arbp*, acidic ribosomal phosphoprotein P0.

Enriched micro RNA (miRNA, miR) preparations from the renal cortices were obtained with the PureLink™ miRNA Isolation Kit (Thermo Fisher, Waltham, MA, USA), and they were reverse-transcribed using the microScript microRNA cDNA Synthesis Kit (Norgen Biotek Corp., Ontario, Canada). The cDNA obtained was diluted and used as a template in a qPCR reaction to analyze the relative expression of several rat miRNAs associated with renal damage: rno-miR-21, rno-miR-132, rno-miR-200c, and rno-miR-423. Both the 5p and 3p arms of each miRNA were determined, and the results were normalized to 5S ribosomal RNA (5S rRNA) and to a calibrator. The primer sequences are presented in Table 2.

Micro RNA gene	Sequence ID*	Primer sequence
rno-mir-21	NR_031823.1	
rno-mir-21-5p		TAGCTTATCAGACTGATGTTGA
rno-mir-21-3p		CAACAGCAGTCGATGGGCTGTC
rno-mir-132	NR_031878.1	
rno-mir-132-5p		ACCGTGGCTTTCGATTGTTACT
rno-mir-132-3p		TAACAGTCTACAGCCATGGTCG
rno-mir-200c	NR_031915.1	
rno-mir-200c-5p		CGTCTTACCCAGCAGTGTTTG
rno-mir-200c-3p		TAATACTGCCGGGTAATGATG
rno-mir-423	NR_032276	
rno-mir-423-5p		TGAGGGGCAGAGAGCGAGACTTTT
rno-mir-423-3p		AGCTCGGTCTGAGGCCCCTCAGT
5S ribosomal RNA	NR_033176.2	CCCGATCTCGTCTGATCT

Table 2. Primer sequences used for the detection of rat micro RNAs by real-time PCR.

* NCBI Reference Sequence number.

2.4. Immunoblotting

Total protein from the renal cortex was extracted by treatment with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, and protease inhibitor cocktail, Sigma-Aldrich) and was quantified using a BCA Protein Assay reagent. Western-blots were performed by overnight incubation of PVDF membranes with polyclonal antibodies raised against rat Klotho, MDR1, AQP3 (Biorbyt, Cambridge, UK), and GSTO1 (Thermo Fisher), at concentrations of 3.3, 2, 0.5, and 1.8 ug/mL, respectively. The signal was detected by chemiluminescence and quantified using a Versadoc MP system (BioRad Laboratories, Redmond, WA, USA).

2.5. Statistical analyses

The comparison between groups was performed by one-factor analysis of variance (ANOVA) followed by a Dunnet's multiple comparison test, using GraphPad Prism 5. Data are presented as the mean ± SE. The level of significance was considered to be $P < 0.05$, and $P < 0.1$ was considered a trend.

3. Results

3.1. Arsenic intake

No overt signs of toxicity were observed throughout the study, and there were no differences in the weight gain between groups at the end of the experiment. Water intake (measured every two days and averaged over 10 days) was less in the 10-ppm group than in the control group (32.7 ± 1.8 mL vs 65.4 ± 19.6 mL), although it did not reach a statistical significance. However, that lower water intake resulted in a lower than expected arsenite intake in the 10-ppm group. The average arsenite intake calculated per rat per day is presented in Table 3. Accordingly, the twenty-four hour urine volume was also less in the 10-ppm group than in the other groups ($P < 0.05$, Table 3).

	Control	1 ppm AsIII	5 ppm AsIII	10 ppm AsIII
Calculated AsIII intake (µg/rat/day)	0	56.3 ± 9.7	267.4 ± 34.4	326.4 ± 17.7
Water intake (mL/rat/day)	65.4 ± 19.6	56.3 ± 9.8	53.5 ± 6.9	32.7 ± 1.8
Plasma parameters				
Creatinine (mg/dL)	0.45 ± 0.02	0.41 ± 0.02	0.44 ± 0.02	0.37 ± 0.03
Pi (mM)	1.50 ± 0.2	1.75 ± 0.1	1.82 ± 0.05	1.68 ± 0.11
Urea (mg/dL)	38.7 ± 3.2	31.9 ± 6.5	33.0 ± 3.1	35.2 ± 4.4
Urinary parameters				
24-hour urine volume (mL)	12.7 ± 0.8[a]	13.5 ± 0.1[a]	11.9 ± 1.0[a]	8.6 ± 0.1[b]
Creatinine (mg/dL)	66.9 ± 12.4	56.4 ± 4.0	72.3 ± 8.1	80.7 ± 10.9
Pi/Creatinine	0.45 ± 0.10	0.38 ± 0.08	0.46 ± 0.04	0.46 ± 0.11
Urea/Creatinine	0.048 ± 0.008	0.043 ± 0.009	0.043 ± 0.009	0.043 ± 0.006
Protein/Creatinine	0.18 ± 0.03	0.16 ± 0.03	0.19 ± 0.01	0.15 ± 0.03
GFR (mL/min)	1.10 ± 0.10	1.12 ± 0.17	1.07 ± 0.03	1.14 ± 0.15

Table 3. Urinary and plasma parameters in rats treated with 0 (control), 1, 5, and 10 ppm of sodium arsenite for 10 days.

Means with different superscripts are different, $P < 0.001$.

3.2. Effects of exposure on the transport and metabolism of arsenicals

The gene expression of proteins involved in the metabolism and transport of arsenicals in the cells was evaluated by real time RT-PCR. Arsenite treatment did not affect the gene expression of the main enzyme that catalyzes the methylation of arsenite, *As3mt*, while it did reduce the expression of another relevant enzyme involved in the detoxification of arsenicals, *Gsto1* ($P < 0.05$, Fig. 1). As for the proteins involved in the influx of arsenicals, arsenite did not change the expression of *Glut1* and *Aqp7*, but it did reduce the expression of *Aqp3* to a similar extent at all doses ($P < 0.05$, Fig. 1). Arsenite treatment distinctly affected the proteins participating in the efflux of arsenicals from the cell: it reduced *Mrp4* at all concentrations ($P < 0.05$), and the same trend was observed in *Mrp1* at 5 and 10 ppm ($P < 0.1$), while the gene expression of *Mrp2* remained unchanged. The two isoforms of *Mdr1* (a and b) were measured:

Arsenite did not affect *Mdr1a* mRNA expression, but it tended to reduce *Mdr1b* at 1 and 10 ppm (P < 0.1, Fig. 1).

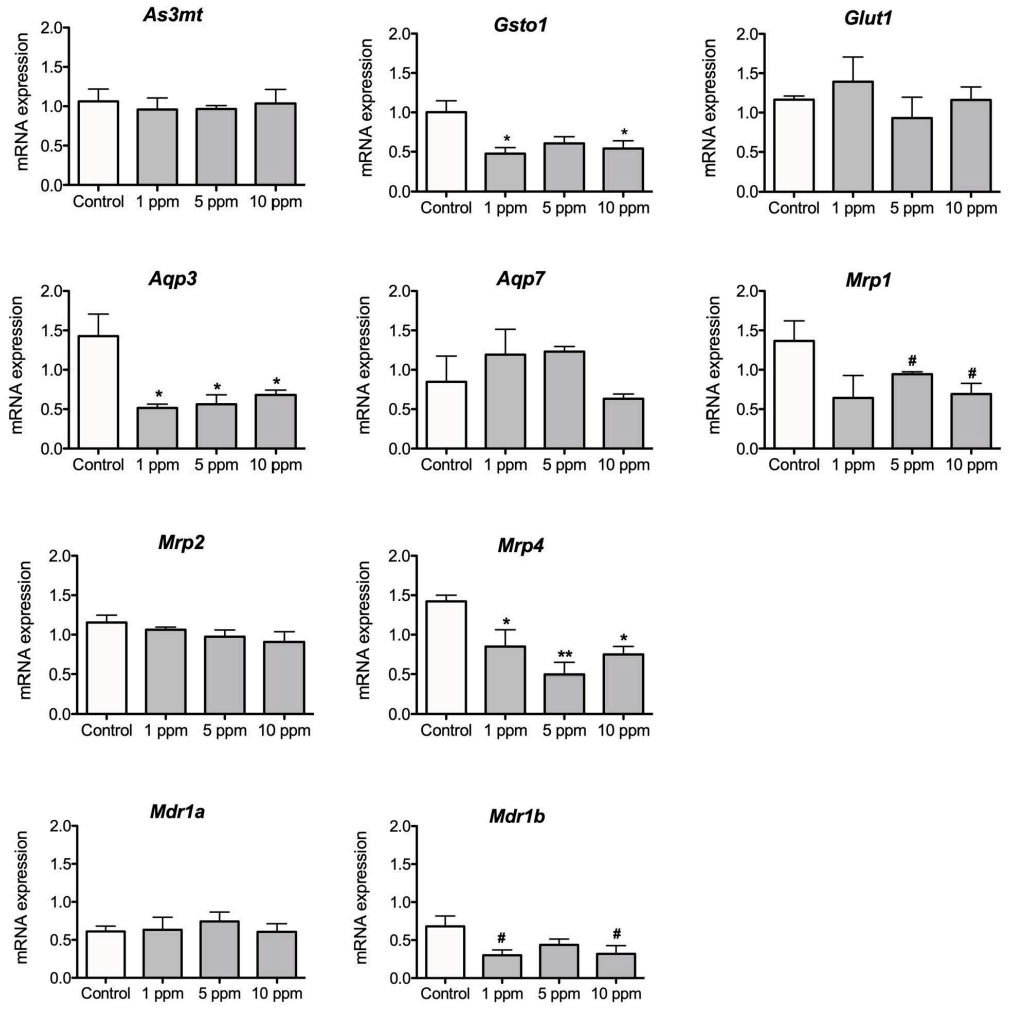

Figure 1. Effect of arsenite on the gene expression of proteins involved in arsenic metabolism. Rats were treated with 0 (control), 1, 5, and 10 ppm of sodium arsenite in water for 10 days. Gene expression (relative to *Arbp*) in renal cortex. Asterisks indicate difference from the control after Dunnet's posttest (*P < 0.05, **P < 0.01, #P < 0.1). *As3mt*, arsenite methyltransferase; *Aqp3 and 7*, aquaporin 3 and 7; *Glut1,* glucose transporter 1; *Gsto1*, glutathione S-transferase omega 1; *Mrp1, 2, and 4,* multidrug resistant proteins 1, 2, and 4; *Mdr1,* multidrug resistant protein/P-glycoprotein.

To assess if the changes observed in gene expression were parallel to protein expression, Western blots of some of the AsIII-altered genes were carried out in protein extracts from the renal cortex. The protein abundance of GSTO1 tended to decrease with 1 ppm AsIII ($P = 0.8$) and was significantly reduced at 5 and 10 ppm ($P < 0.05$, Fig. 2). Similarly, AQP3 was also reduced at 5 and 10 ppm ($P < 0.05$), while no effect could be detected on MDR1 protein expression (Fig. 2).

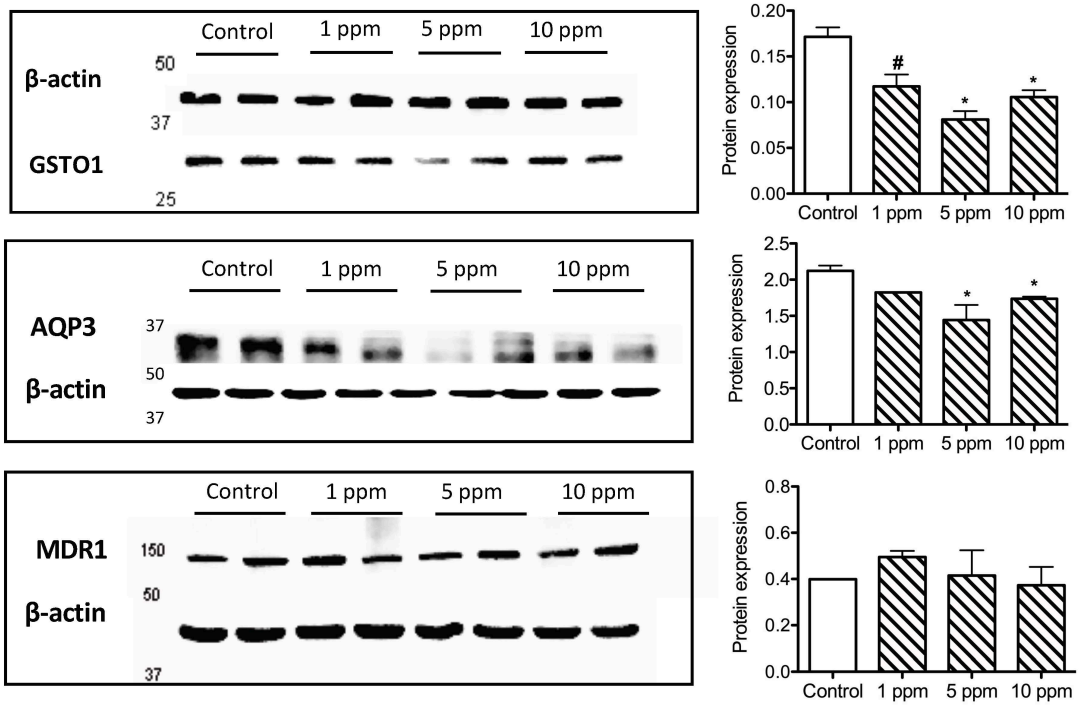

Figure 2. Effect of arsenite on the abundance of proteins involved in arsenic metabolism. Rats were treated with 0 (control), 1, 5, and 10 ppm of sodium arsenite in water for 10 days. Representative Western blots of selected proteins (relative to β-actin). Asterisks indicate difference from the control after Dunnet's post test (*$P < 0.05$, $^{\#}P < 0.1$). AQP3, aquaporin 3; GSTO1, glutathione S-transferase omega 1; MDR1, multidrug resistant protein/P-glycoprotein.

3.3. Effects on renal function

The parameters measured in urine and plasma were used to evaluate renal function. No effect by arsenite intake on the concentrations of creatinine, urea, or phosphate was observed, either in the plasma or urine (Table 3). The calculated glomerular filtration rate was also similar between groups. The analysis of blood gases and pH did not provide evidence any alteration due to the arsenite treatment (data not shown).

After treatment with AsIII at 5 and 10 ppm, the urinary concentrations of the two early markers of acute renal damage, NGAL and FABP3, revealed approximate increases of 2- and 7-fold with respect to the control, respectively ($P < 0.05$, Fig. 3A). Four micro RNAs related to kidney damage were determined, in both the 3p and 5p arms. While some of them could not be detected in the renal cortex, we were able to quantify mir-21-5p, mir-200c-3p, and mir-423-3p, revealing that arsenite treatment did not alter the expression of any of them (Fig. 3B).

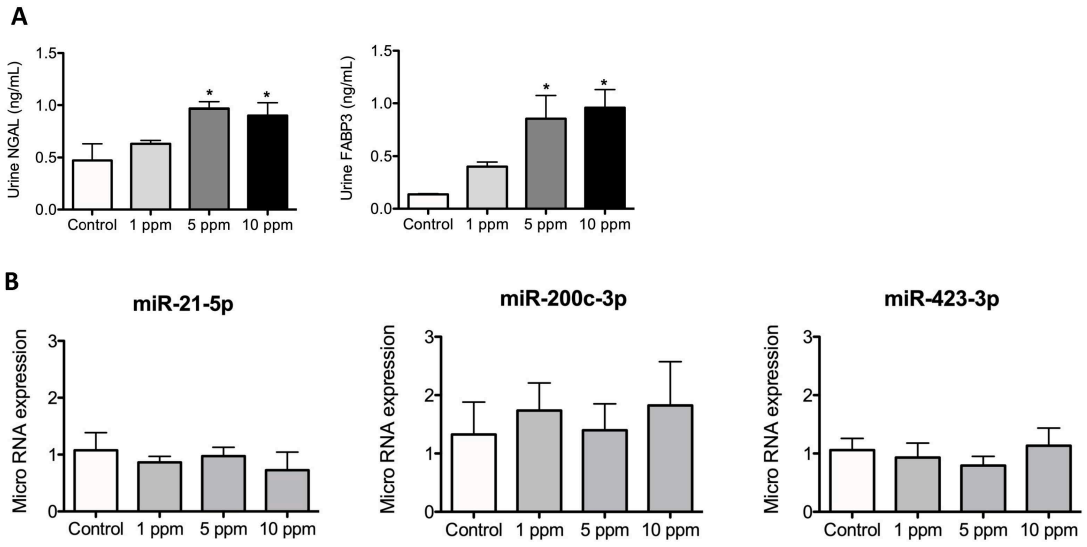

Figure 3. Arsenite causes renal damage. Rats were treated with 0 (control), 1, 5, and 10 ppm of sodium arsenite for 10 days. A, Urinary concentrations of neutrophil

gelatinase-associated lipocalin (NGAL) and fatty acid-binding protein 3 (FABP3). B, MicroRNA expression in the renal cortex (relative to 5S rRNA). Asterisks indicate difference from the control after Dunnet's posttest (*P < 0.05).

3.4. Effects on Pi homeostasis

Despite the apparent absence of an effect by arsenite on the renal handling of Pi, we also checked the effect on the plasma concentrations of several Pi-related hormones: PTH, soluble Klotho, and FGF23. The plasma concentrations of PTH and FGF23 were similar between groups. Klotho plasma levels decreased by 23% in the 1-ppm group (P < 0.05) and by 35% in the 5- and 10-ppm groups (P < 0.01) with respect to the control (Fig. 4A).

Klotho and *Fgfr1* mRNA in the kidney cortex was significantly less abundant in all AsIII-treated groups than in the control group, in a dose-independent manner (P < 0.001 and P < 0.05, respectively, Fig. 4B). No effect of arsenite treatment was observed in the expression of *NaPi-IIa*. Furthermore, the protein expression of Klotho in the renal cortex was decreased by treatment with 5 and 10 ppm AsIII (P < 0.05, Fig 4C).

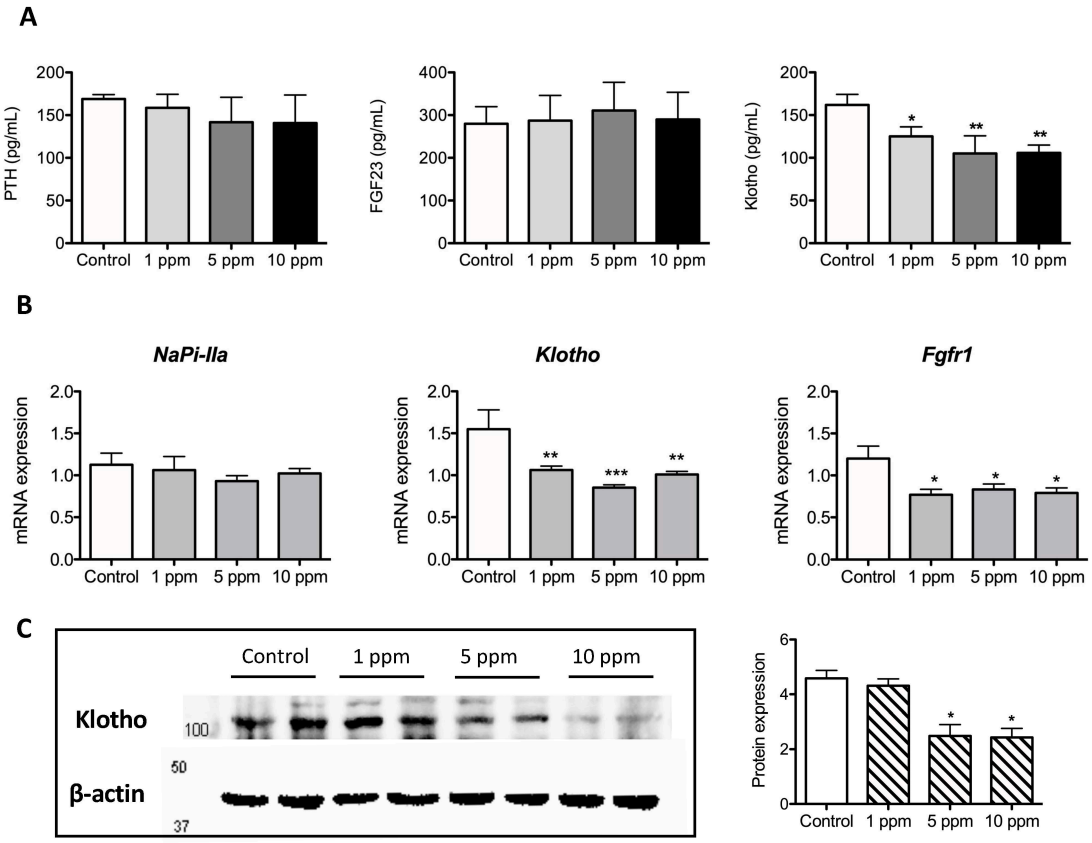

Figure 4. Effect of arsenite on Pi homeostasis. Rats were treated with 0 (control), 1, 5, and 10 ppm of sodium arsenite for 10 days. A, Plasma concentrations of parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and Klotho. B, Gene expression (relative to *Arbp*) in the renal cortex. C, Western blot and intensity histogram of Klotho (relative to β-actin) in the renal cortex. Asterisks indicate difference from the control after Dunnet's posttest (*P < 0.05, **P < 0.01, ***P < 0.001). PTH, parathyroid hormone; FGF23, fibroblast growth factor 23; *NaPi-IIa,* sodium-dependent phosphate transporter type II, isoform a; *Fgfr1*, fibroblast growth factor receptor 1.

4. Discussion

The cytotoxic effects of AsIII have been associated with the metabolism and accumulation of AsIII in the cell. Entry by arsenite into the cell is facilitated by some

aquaporins and hexose transporters (Liu, 2010; Liu et al., 2006). In addition to being permeable to water, aquaporins 3, 7, 9, and 10 –the so-called aquaglyceroporins– allow entry by small nonionic molecules such as glycerol and urea, and they are essential in the homeostasis of metalloids (Bienert, et al. 2008; Liu, 2010). Of these aquaporins, we measured the expression of AQP3 and 7 since they are expressed in the kidney. In our study, the arsenite-treated rats presented 3-fold lower renal expression of *Aqp3* mRNA than the controls, and no effect was observed for *Aqp7* abundance. Accordingly, AQP3 protein expression was also reduced, but the effect was lower than on the RNA and at the highest concentrations of arsenite. Experiments with null mice have shown that AQP3 plays a relevant role in water reabsorption, given that AQP3-null mice presented polyuria and a lower capacity to concentrate urine, whereas AQP7-null mice maintained the concentration capacity but with increased glycerol in urine (Matsuzaki et al., 2017). We did not observe any effect on urine concentration (i.e., urinary creatinine levels were similar), which is consistent with a modest effect on the protein expression of AQP3 under our experimental conditions. On the other hand, due to its involvement in glycerol transport, AQP3 deficiency has also been related to impaired lipid biosynthesis and glycerol metabolism, which ultimately lead to reduced cell proliferation (Michalek, 2016).

The exact participation of AQP3 in the transport of arsenicals is not clear. It has been demonstrated that, in an aqueous solution and at physiological pH, the predominant form of inorganic arsenic is arsenic trioxide ($As(OH)_3$) (Ramírez-Solís, et al. 2004) and that human AQP3 (hAQP3), unlike hAQP7 and 9, does not show a significant uptake of $^{73}As(OH)_3$ when expressed in oocytes (Liu et al., 2004). In mammals, a proportion of arsenite is methylated to mono-, di-, and tri-methylated products, mainly in the liver, but when compared to AQP7, AQP3 also does not seem to play a significant role in the transport of methylated forms (Liu, 2010).

Mammalian hexose transporter GLUT1 (SLC2A1) has also been shown to facilitate the uptake of trivalent arsenicals when expressed in biological systems *in vitro*, and

indeed it is believed to represent the main entrance route for arsenite in erythrocytes and brain epithelial cells, where aquaglyceroporins are not abundantly expressed (Liu et al., 2006). GLUT1 is highly expressed in most tissues, and in the kidney it is localized to the basolateral membrane of the proximal straight tubules (Augustin, 2010). Therefore, we also assessed its possible regulation by arsenite, but none of the AsIII doses used in our study had any effect on *Glut1* mRNA expression.

The efflux of methylated arsenic from the cell occurs primarily through multidrug resistant proteins, members of the ATP-binding cassette (ABC) transporters, sub-families B and C (i.e. MRP1, 2, and 4 and MDR1/P-glycoprotein) (Leslie, 2012). In this study, arsenite treatment reduced the gene expression of *Mdr1b*, *Mrp1*, and *Mrp4* (Fig. 1). Even though the protein level of MDR1 was still unchanged during the 10-day interval, the changes in the level of mRNA expression suggest an impairment in the clearance of arsenical forms out of the cell at longer exposure times. In renal cells, MRP4 and MDR1 are localized to the apical (brush border) membrane, and MRP1 is localized to the basolateral membrane (Launay-Vacher et al., 2006; van Aubel et al., 2002). Therefore, the outflow of arsenicals from the cells would be reduced, consequently enhancing the susceptibility of the cells to the toxic effects of arsenic. Indeed, the number or activity of multidrug resistance proteins has been directly associated with cellular tolerance or sensitivity to the toxic effects of arsenic (Thomas, 2007).

Two isoforms of MDR1/P-glycoprotein have been described for rodents, encoded by genes *Mdr1a* (*Abcb1a*) and *Mdr1b* (*Abcb1b*), and under certain circumstances they have been shown to play dissimilar functions in different organs or to respond differently to the same stimulus (Koehn et al., 2019; Su et al., 2017; Zhang et al., 2019). In our study, only *Mdr1b* RNA was affected by the AsIII treatment, whereas *Mdr1a* remained unchanged. Coincidently, *Abcb1b* mRNA also decreased in the renal cortices of rats exposed to vitamin D deficiency, with no changes observed in *Abcb1a*

(Wang et al., 2015), but the physiological relevance of this unequal regulation between both isoforms has not been explained.

The detoxification of arsenic in the cell involves several reduction and methylation reactions and subsequent efflux out of the cell. The methylation of the trivalent oxidation states of arsenic is mainly catalyzed by AS3MT (Lin et al., 2002), which produces methylated pentavalent arsenicals that are substrates for the reducing activity of GSTO1 (Zakharyan et al., 2001). We have found that treatment with arsenite does not alter the gene expression of renal *As3mt*, whereas it does reduce both *Gsto1* gene expression by around 50% in a dose-independent manner (Fig. 1) and protein abundance (Fig. 2). We did not measure the activity of the enzyme, but treatment with 5 mg/kg arsenate during 4 weeks reduced the renal glutathione-S-transferase activity in rats (Miltonprabu et al., 2017). This could imply the accumulation of methylated pentavalent forms inside the cell, thereby causing nephrotoxicity.

To our knowledge, this is the first time that alterations in the proteins involved in arsenical metabolism due to arsenite treatment *in vivo* have been reported. These results shed light on the possible mechanisms by which arsenite causes nephrotoxicity, and they also have far-reaching implications for therapeutic purposes. Multidrug resistant proteins in the renal tubules participate in the urinary excretion of a myriad of organic molecules, including xenobiotics and drugs, and therefore the implications of the alterations caused by arsenite described here extend to pharmacokinetics, drug efficacy, and the manifestation of adverse effects.

Given that the changes exerted by arsenite in the detoxification system of renal cells indicated that the handling and elimination of arsenicals was impaired, we decided to evaluate the possible renal damage. Under the present conditions of dose and exposure time, arsenite treatment did not affect the plasmatic concentrations of creatinine or urea or the urinary levels of creatinine, urea, or protein. Consistently, the glomerular filtration rate was similar between groups. According to these results, major renal damage could be ruled out. This is in contrast to some works clearly showing

renal damage by arsenic in humans (Meliker et al., 2007, Robles-Osorio et al., 2015), even in rodents (Shahid et al., 2014, Riaz et al., 2020), but the experimental design and epidemiology in humans have, sometimes, provided conflicting results (Samelo et al., 2020). However, it is known that these classical endpoints are not sensitive enough to detect early renal damage (Sabbisetti and Bonventre, 2012), and several molecules occurring in urine in the very early stages of renal injury have been found to be useful as biomarkers, such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), N-acetyl-β-D-glucosaminidase (NAG), liver fatty acid binding protein (L-FABP), heart fatty acid binding protein (H-FABP, also known as FABP3), tissue inhibitor metalloproteinase-2 (TIMP-2), insulin-like growth factor binding protein 7 (IGFBP-7), clusterin, and netrin, among others (Griffin et al., 2019; Schaub, 2016). We have shown that these biomarkers also apply to the renal damage caused by arsenite and that rats treated with 5 and 10 ppm of arsenite had increased concentrations of both of NGAL and FABP3 in urine when compared to non-treated rats. NGAL (also known as lipocalin 2) is a 25-kDa glycoprotein that is present in plasma and is bound to metalloproteinase-9 from neutrophils. It is also expressed at very low levels in various tissues of the body, but it is readily inducible by injury in epithelial cells, including those from the kidneys, liver, and lungs (Mishra et al., 2003). Plasma NGAL is filtrated in the glomerulus and is reabsorbed in the proximal tubules, and therefore any urinary excretion of NGAL is assumed to be derived from a proximal tubule injury that precludes reabsorption and/or increases *de novo* synthesis (Devarajan, 2010). Urine NGAL is among the most promising early biomarkers of acute kidney injury, preceding the appearance of other urinary markers (Griffin et al., 2019). Fatty acid binding proteins, in turn, are nine 15-kDa molecules localized in the cytoplasm of almost all cell types. Heart fatty acid binding protein (or FABP3) is abundantly expressed in cardiomyocytes and, to a much lesser extent, also in muscles, distal tubular renal cells, the brain, the mammary glands, and the placenta. Unless there is an ongoing cardiomyopathy, the presence of FABP3 in urine reflects distal

tubular damage (Pelsers, 2008), and it has proved to be a good marker of early renal injury (Hofstra et al., 2008; Kokot, et al. 2014). In a rat model of diabetic nephropathy, FABP3 showed an early and dramatic rise together with other early biomarkers (Alter et al., 2012).

Several micro-RNAs have also been used as early biomarkers of renal damage. Micro-RNAs are short (~22 nucleotides), noncoding RNA molecules that have regulatory functions over the expression of their multiple target messenger RNAs (Bartel, 2004). They participate in multiple biological processes and have also been related to several pathological conditions, including those that affect the kidney (Bhatt et al., 2016; Ichii and Horino, 2018). In a screening study of more than 1800 urinary miRNAs, miR-21, miR-200c, and miR-423 were identified as the most sensitive markers of acute kidney injury in human patients (Ramachandran et al., 2013). In a rat tubular injury model induced by ischemia/reperfusion, miR-21 was upregulated in kidney cortices (Saikumar et al., 2012). By contrast, we have found no effect of arsenite treatment on the expressions of miR-21, miR-200, and miR-423. Likewise, in a study performed on a population of children exposed to arsenic, Cárdenas-González et al. (2016) did not find any association with these three miRNAs, even if they showed signs of early renal damage (i.e., increased KIM-1 levels). Therefore, the circumstances that lead to the upregulation of these miRNAs in renal damage are still to be elucidated, as it is the role of other miRNAs and their corresponding target proteins.

Arsenite treatment significantly reduced soluble Klotho at all doses (Fig. 4A). *Klotho* mRNA expression in the renal cortex paralleled the Klotho plasma concentrations, and it was also accompanied by a reduction in the renal concentration of Klotho protein, although this effect was only observed in the group treated with the highest concentration of arsenite (Fig. 4B and C). This is in accordance with the lower Klotho plasma concentrations, given that the kidney is thought to be the major source of soluble Klotho in rodents. It also plays an active role in the development of renal

injury. A study by Hu et al. (2010) found that a drop in Klotho levels in plasma and the kidney preceded an increase in NGAL and that this parameter was the first one to change after ischemia-reperfusion injury in rats. Moreover, not only is antiaging Klotho a useful marker, it also prevents renal damage, which is exacerbated when the Klotho concentration is reduced (Hu et al., 2012).

Overall, according to the observed results showing constant creatinine, conserved GFR, and high urinary biomarkers (i.e., NGAL and FABP3), the pathophysiological condition of these arsenite-treated rats would be early tubular damage, both proximal and distal, without excretory dysfunction (for instance, subclinical acute kidney injury) (Desanti De Oliveira, et al. 2019; Schaub, 2016).

Acute kidney injury and chronic kidney disease are associated with alterations of the renal handling of phosphate, which ultimately leads to hyperphosphatemia, so we set out to investigate if the phosphate balance was disrupted. In our study, the arsenite treatment did not alter either phosphatemia or the renal gene expression of the phosphate cotransporter *NaPi-IIa*, which is probably because of the early stage of renal injury caused by the arsenite.

Nevertheless, we wanted to investigate the endocrine milieu that ultimately controls phosphate homeostasis. Both PTH and FGF23 are phosphaturic hormones, and an increase in their concentrations in plasma is a constant observation in renal failure, both in human patients and animal models (Jacquillet and Unwin, 2019). In this work, PTH and FGF23 were not affected by arsenite treatment at any dose, consistent with normophosphatemia in the animals of this study.

FGF23 actions are exerted in the kidney through the FGFR1 receptor, but only after the binding of FGF23 to Klotho (Urakawa et al., 2006), so target tissue sensitivity should be assessed in terms of both the specific receptor expression and Klotho levels, which are reduced upon arsenite exposure. It is notable that the gene expression of *Fgfr1* was also consistently reduced by arsenite, which, in conjunction with the lower Klotho concentrations, could imply a resistance of kidney tubular cells to FGF23. On

the other hand, the detailed repercussions of the lower expression of renal FGFR1 are immeasurable, yet they definitely exceed phosphate homeostasis, given that fourteen of the eighteen FGFs bind FGFR1, most of them without the need for the co-receptor Klotho (Helsten et al. 2015).

5. Conclusion

The interpretation of this set of data is complex, since there are many players in the detoxification of arsenical forms, with varying contributions depending on several aspects, such as the degree of methylation and/or the glutathione conjugation of the arsenical and several other levels amenable to regulation (e.g. epigenetic marks, post-transcriptional or post-translational changes, enzymatic activity, etc.). However, the results presented here support the influence of arsenite on its particular detoxification system, which in general terms leads to lower metabolism and lower clearance out of the renal cell. This could lead to the accumulation of arsenical forms in the cell, thereby increasing cytotoxicity, which is reflected by the increase in the early kidney damage markers (i.e., NGAL, FABP3) observed in our study.

Authors contributions

C. Sosa: Writing - original draft, Writing - review & editing, Methodology, Investigation, Formal analysis. N. Guillén and S. Lucea: Investigation, Writing - review & editing. V. Sorribas: Conceptualization, Funding acquisition, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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