IMPLEMENTATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF INORGANIC ARSENIC SPECIES IN OCCUPATIONALLY EXPOSED HUMAN URINE SAMPLES AND ITS TOXIC EFFECTS ON EPITHELIAL CELLS OF RENAL COLLECTING TUBULE

NICOLE ROLDÁN[1,2], NICOLÁS SALINAS-PARRA[2], ALEXIS A. GONZALEZ, FLAVIA CIFUENTES-ARANEDA[2], HELEN ARIAS[1], MANUEL BRAVO[1] AND WALDO QUIROZ[1]

1) Laboratorio de Química Analítica y Ambiental, Pontificia Universidad Católica de Valparaíso, Avenida Universidad 330 Curauma, Valparaíso, Chile
2) Laboratorio de Química Biológica, Pontificia Universidad Católica de Valparaíso, Avenida Universidad 330 Curauma, Valparaíso, Chile

ABSTRACT

In this study a method was implemented for the determination of total As and its inorganic species in human urine by HPLC-HG-AFS. The method was applied to workers occupationally exposed to mining and thermoelectric operations. Results shows high levels of As in urine up to 188±4 µg L^{-1} and As(III) up to 10.7±0.6 µg L^{-1}. Toxic effects of As(III) at the concentration levels founded in human urine were evaluated over the morphology and fibrosis of renal epithelial collecting duct cells in vitro. Our results suggest that urinary tract epithelial renal tubular cells exposed to this concentration of As(III) result in epithelial-mesenchymal transition.

Keywords: Arsenic; speciation; renal cells; urine; toxic effects

INTRODUCTION

Arsenic (As) is an element that is considered to be hazardous to human health [1]. The terrestrial abundance of arsenic is approximately 1.5 to 3 mg kg^{-1}[2]. Although As is found in weathering of rocks and introduced to the environment by volcanic activity and natural combustion of fossil materials,[3] it is also found as an anthropogenic product. Anthropogenic sources include pyrometallurgical processes, the use of fossil fuels, copper smelting processes and coal combustion; the amount of As released as a result of these processes exceed natural sources by 3:1[3].

Humans are exposed to many different forms of inorganic and organic As species present in the environment. There are two predominant inorganic forms of As: As (III) and As (V). Vega et al, [4] reported the toxicity order of arsenic species to be (Inorganic Arsenic III) iAs^{III}> monomethylarsine oxide ($MMAO^{III}$) > (Dimethyl Arsenic III) DMA^{III}> (Dimethyl Arsenic V) DMA^{V} > (Mono methyl Arsenic V) MMA^{V} > (Inorganic Arsenic (V)) iAs^{v}. Notably, As(III) is much more toxic, soluble and mobile than the pentavalent species[2,5].

The World Health Organization (WHO) set the maximum concentration of As in drinking water to 10 µg L^{-1}[6]. Following this recommendation, individuals who consume approximately 2 L of water would ingest a maximum of approximately 20 µg of As daily[7]. As is absorbed by the lungs and the gastrointestinal tract and is excreted in the urine, normally within 1–2 days. Measurements of urinary As levels are considered to be the most reliable and accurate markers of acute As exposure[8]. Indeed, such measurements have been found to correlate well with exposure levels of populations that live near industrial point sources[9]. Importantly, urinary As concentrations may reflect As concentrations found in the intratubular lumen of the ureter bud and renal collecting ducts[10] where the urine is subjected to the physiological process of concentration. Then, epithelial cells may be in direct contact with As, leading to its deleterious effects.

Exposure to As has been associated with nephrotoxicity, the development of interstitial fibrosis and tubular epithelial-mesenchymal transitions. Proliferative disorders and fibrotic lesions are characterized by the augmented expression of CTGF (Connective Tissue Growth Factor) and epithelial mesenchymal transition phenotypes[11]. The induction of fibrosis and epithelial mesenchymal transition leads to deterioration of renal function[12].

The tubular epithelial cells in the kidney are exposed to filtered and excreted substances, which are concentrated in the distal part of the nephron called collecting duct. Studies linking the presence of As with kidney damage have not produced clear conclusions regarding the damage level of fibrosis generated by the specific presence of As(III) in epithelial renal tubular cells at the concentrations found in the urine for workers who are occupationally exposed to As.

The aim of this work was to implement an analytical method to determine the content of inorganic arsenic species in the urine of workers exposed to As through mining activities. We also aimed to evaluate the effect of As(III) on morphology and fibrosis in renal epithelial collecting duct cells in vitro at the concentration levels founded in human urine.

EXPERIMENTAL

Standard solutions and reagents

All working solutions were prepared using high purity (18 Mohm) deionized water from a NANO pure system (Barnstead S.A.). Standard solutions of As (III), As (V), MMA and DMA were prepared from using $NaAsO_2$ (98% m/m Merck), $Na_2HAsO_4 \cdot 7H_2O$ (98% m/m Aldrich), $CH_3AsO(ONa)_2 \cdot 6H_2O$ (98% m/m Aldrich) and $(CH_3)_2As(O_2)Na \cdot 3H_2O$ (98% m/m Sigma) salts, respectively. These standard solutions were stored at 4 ° C until further dilution.

The acids used for digestion were HNO_3 (65% w/v, suprapure grade) and H_2O_2 (30% w/v); both acids were purchased from Merck (Darmstadt, Germany).

The reducing reagents used were KI (p.a. ISO Merck), ascorbic acid ($C_6H_8O_6$; ACS ISO Merck), $NaBH_4$ (analytical reagent grade Merck, Darmstadt, Germany) and NaOH (analytical reagent grade Merck, Darmstadt, Germany). The mobile phases were prepared from the respective salts: $(NH_4)_2HPO_4$ and $(NH_4)H_2PO_4$ (p.a. Merck).

All materials used to prepare the different solutions, either glass or polyethylene, were rigorously washed with water and immersed in deionized water and HNO_3 10% v/v for 24 hours. Then, the materials were rinsed with purified water, dried and stored in plastic bags for later use.

The solutions used for cell culture were autoclaved subsequent to their preparation and were then filtered and stored at 4°C.

500 mg L^{-1} solutions of the As (III) standards were prepared from $NaAsO_2$ (Sigma-Aldrich 90%) in deionized water and stored in a polypropylene flask at 4°C. Lower concentration standards were prepared daily by diluting this concentrated solution.

Cell incubation experiments

Cell line culture and treatments. M-1 cortical collecting duct cells (American Type Culture Collection; CRL-2038) were grown in DMEM-F12 media supplemented with 10% FBS, 5 µM dexamethasone, 1X insulin-transferrin-selenium, and 100 U/ml penicillin/streptomycin in a humid atmosphere of 5% CO_2-95% ambient air at 37°C. The cells were then treated with As (III) (1, 10 and 100 nM) for 16 h.

Immunofluorescence studies in M-1 cells. Subconfluent M-1 cells (30–40%) cultured in chamber slides (Nalge Nunc, Rochester, NY) were fixed in cold methanol for 20 min, blocked with PBS-Tween (0.1%) plus BSA (3%) for 1 h, and stained with rabbit anti-β-actin (Cat. no. sc-130657; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilutions. The cells were imaged using Alexa Fluor 488 conjugated to anti-rabbit IgG (Invitrogen, Carlsbad, CA) at a 1:1000 dilution. Samples were counterstained with 4,6-diamidino-2-phenylindole (Invitrogen). Negative controls were obtained by omission of the

e-mail:waldo.quiroz@pucv.cl

specific primary antibody.

Changes in the morphology of M1 cells treated with As (III). Images were acquired on a Nikon Eclipse E200 microscope equipped with a halogen lamp and a high-luminescence white LED illuminator. The images were obtained at 40X magnification. The cells were photographed with a NIKON digital sight DS-U3 digital camera using a blue filter (360/40 nm excitation and 460/50 nm) and a green filter (480/40 nm excitation and 535/50 emission nm). To evaluate the change in lengths of the cells treated with As (III), morphometric analyses were performed using the Image J program. Briefly, based on nuclei staining (DAPI-blue), a line was drawn from the center of the nuclei to the edge of the cells, and the length was measured in microns. Values are presented as the cell length of 3 microscope fields.

Protein expression of CTGF. Protein expression levels were quantified after immunoblotting using a 1:250 dilution of the specific antibodies: Primary antibody were followed by incubation with either donkey anti-Goat IgG IRDye 800 CW (Li-cor Biosciences, Lincoln, NE) at a 1:1000 dilution. Densitometric analyses were performed by normalization against β-actin.

Statistical analyses of immunoblots and cell morphology. Each experiment constituted an average of five to six independent observations (each well represented an independent observation). Experiments were performed in at least three different cell passages. Cells were used until passages 10–12. Differences between the sample groups were assessed by one-way ANOVA followed by Tukey's test using GraphPad Prism software v 5.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant. The results shown in the plots and texts are expressed as the mean ± SD.

Determination of total As and its speciation

Urine samples were treated according to a method previously reported by our group for total Sb determination[13]: Briefly urine and culture media were stored in 2 mL Eppendorf tubes at -20°C for further digestion. Open digestion was performed by adding 3 mL of concentrated HNO_3 to the sample in an 80 mL glass flask and heating the mixture to 100°C. After the acid evaporated, 1 mL of water was added 3 times to remove traces of nitric acid. When the process was complete, the resulting digest was transferred to a 10 mL volumetric flask and stored at 4 ° C for further analysis.

Determination of total As levels in urine and culture media digest was performed by standard addition. The working conditions and solutions used are presented in table 1.For this, 200 µg L^{-1} standard solutions of As(III) were prepared daily from 1 mg L^{-1} As (III) solutions.

To each volumetric flask, a standard solution of As(III) was added and mixed with an amount of urine or culture media and was subsequently digested with reducing reagents (KI/ascorbic acid and concentrated HCl). The final volume was adjusted to 50 mL with distilled, deionized water, and the sample mixtures were stored at room temperature for 12 hour to complete the reduction reaction.

Table 1. Experimental conditions for the determination of total As content in urine by HG-AFS.

Variables	Conditions
Pre-reduction conditions	KI 1.5% m/v; ascorbic acid 6% m/v (1 hour)
Linear Range	50-1000 ng L^{-1}
Hydride generation solution	HCl 1.5 mol L^{-1}; $NaBH_4$ 0.75 % m/v in NaOH 0.4 % m/v
Ar flow	300 cm^3 min^{-1}
Supplementary hydrogen flow	20 cm^3 min^{-1}

Arsenic speciation analyses were performed using an HPLC coupled to an HG-AFS detection system. Chromatographic separation conditions were based in the works previously reported Bohari et. al [14, 15] and are provided in table 2.

All samples used for the speciation analysis of As within predetermined time periods were initially filtered and kept refrigerated at 4°C prior to analysis. Then, the samples were filtered through 0.22 µm filters.

To facilitate post-column UV oxidation, a T-junction was inserted at the exit of the column Teflon tube through which a $K_2S_2O_8$ solution was circulated; the solution was driven by a peristaltic pump at 0.5 mL min^{-1}. Teflon tubing was wrapped around a lamp of 44 cm Hg (15 W) and connected to another T-junction and ultimately to the HG-AFS system.

Table 2. Summary of conditions used for an HPLC coupled to a HG-AFS detection system for the determination of As species.

HPLC (Jasco HPLC system PU-2089S Plus)	
Column	Hamilton PRPX-100 (250 x 4.1 mm id, particle size 10 µm)
Mobile phases (mmol L^{-1})	A: $(NH_4)H_2PO_4$ 2 mmol L^{-1} pH 4.6 B: $(NH_4)_2HPO_4/(NH_4)H_2PO_4$ 30 mmol L^{-1} pH 7.6
Gradient elution program	A: 0-3 min B: 3-9 min
	A: 9-20 min
Flow rate (mL min^{-1})	0.8
Injection volume (µL)	100
Detection HG-AFS (PS Analytical Ltd, Millennium Excalibur system)	
As BDHCL	
Primary current (mA)	27.5
Boosted (mA)	35
HCl (mol L^{-1})	1.5
HCl flow (mL min^{-1})	0.35
$NaBH_4$ (% m/v)	0.4 (in 0.4% m/v NaOH)
Flow $NaBH_4$ (mL min^{-1})	0.35
Argon flow (primary) (mL min^{-1})	300
Hydrogen (auxiliary) (mL min^{-1})	40-50

RESULTS

Total As concentration in urine samples

Certified Reference Material NIES Nº18 Human Urine was used to validate the methodology for the determination of total arsenic concentrations in urine, which resulted in a certified total As concentration of 134 ± 11 µg L^{-1}. This reference material was reconstituted as recommended by the manufacturer by adding 9.57 g of deionized water to the lyophilized urine simple. Then, 2 ml of this solution was analyzed after digestion under standard additions using HG-AFS as a detection system. The total As concentration was determined to be 138 ± 6 µg L^{-1}. This result established that the methodology was able to yield reliable results.

The methodology was applied to urine samples of residents or workers in the area of Puchuncaví Valley in Chile, an area contaminated by mining and thermoelectric power generation byproducts[16]. These samples were taken on the basis of a sampling plan previously published in our previous work [17]. A total of 8 urine samples were collected, and their total As concentrations with pH and creatinine parameters are shown in table 3.

Table 3. Urine analyses from occupationally exposed workers from Puchuncaví Valley in Chile.

Sample	pH	µg As g^{-1} creatinine	µg As L^{-1}
A	4.77	20.5±0.2	31±5
B	4.95	26.6±0.2	30.6±0.6
C	4.87	50.0±0.1	125±10
D	5.07	24.3±0.3	17±4
E	6.47	24.6±0.1	27.1±0.7
F	5.5	31.1±0.1	34.2±0.7
G	6.67	76.1±0.1	51±3
H	4.54	85.9±0.2	188±4

The biological exposure index recommended by the ACGIH for As (inorganic arsenic + methylated metabolites) in workers is 35 µg L^{-1}[18], and three samples were observed to have As concentrations above this level (C, G

and H). Normal values for total As concentration in urine vary from 13 to 46 µg L^{-1}. Hwang et al previously determined the total As in urine samples of a control group to be 27.4 ± 17.7 µg L^{-1} [19]. Thus, considering the interval between 10 and 45 µg L^{-1} as normal values for the population, it can be concluded that five of the analyzed samples had concentrations that were considered normal, and three samples had concentration values located above this level (C, G and H).

Arsenic speciation in urine

After determining the total content of As in urine, we proceeded to analyze the speciation of this As. First, the stability of As species in relation to storage periods was studied for 4 arsenic species, As (III), DMA, MMA and As (V), through a recovery study. Urine samples were fortified simultaneously with 10 µg L^{-1} of each arsenic species, and the concentration of each sample was determined after 7 days of storage. Chromatograms of Figure 1 clearly show that the storage time did not greatly affect the recovery of the As species. These results coincide with those reported by Feldmann et al, who reported that the As species were stable for up to 2 months[20]. However, Figure 1 also shows a clear resolution problem in the separation of DMA/MMA, so we chose to express these species as a sum based on the total area of their signals.

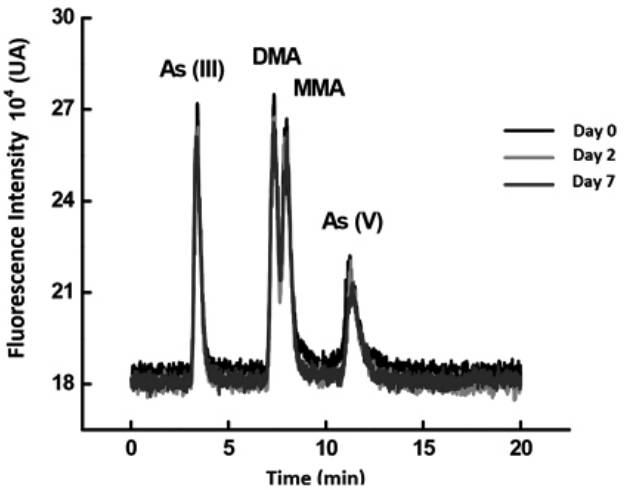

Figure 1. Separation of anionic arsenic species in 10 µg L^{-1} spiked urine samples during a storage period of 7 days.

An As speciation methodology was applied to identify the As species in 8 urine samples. Chromatograms of all the samples are shown in Figure 2. Table 4 shows the concentration of each As species in these samples.

Table 4. Total As (µg L^{-1}) and anionic As species concentrations in urine samples from workers occupationally exposed to As.

Samples	As(III)	DMA+ MMA	As(V)	∑As species	Total As
A	<2	31±1	2.3±0.2	35±1	31±5
B	2.1±0.6	25±1	<2	29±1	30.6±0.6
C	10.7±0.6	111±4	3.5±0.1	125±4	125±10
D	<2	7.5±0.3	<2	9.4±0.3	17±4
E	2.1±0.2	14.3±0.9	<2	17.6±0.9	27.1±0.7
F	2.0±0.1	20±1	2.0±0.1	24±1	34.2±0.7
G	8.6±0.4	29.4±0.2	5.1±0.1	43.1±0.5	51±3
H	6.6±0.4	182±2	2.4±0.1	191±2	188±4

Limit of Quantification (LOQ) 2 µg L^{-1} for As(V) and As(III)

As seen in table 4, the majority of the species were DMA+MMA, representing approximately 80% of total species in most of the analyzed samples; the inorganic species As(III)+As(V) accounted for approximately 20% of the total species. These results are in agreement with previously reported results indicating that organic species were mainly excreted through urine [21, 22].

Moreover, in 6 of the 8 samples, it was possible to quantify As(III). The concentration values ranged between 2.1 a 10.7 µg L^{-1} with an average of 4.4 µg L^{-1}, and these values were higher than previously reported averages for exposed workers in the semiconductor industry[19]

Considering that As(III) is the most toxic As species, we were interested in investigating the possible effects of As(III) for kidney cells at concentration levels determined in this study. To this end, the effects of As(III) on epithelial cells of rat kidneys were evaluated at nM levels.

Distribution of As(III) in M-1 cells

Table 5 shows the extracellular and intracellular levels of As(III). As(III) treatment at 100 nM resulted in a significant increase in intracellular As(III) concentrations (89 ± 5 nM), which corresponded to 89% of the total concentration added. The percentages of As(III) uptake into cells at 10 and 100 nM of As(III) were similar; however, the cell morphology was affected at 100 nM (Figure 4). Our results suggest that As(III) is able to enter cells after 16 h of treatment. In contrast, Catalayud et al.[23] demonstrated that As(III) retention in colon cancer cells (caco-cells) was 0.9-2.4%.

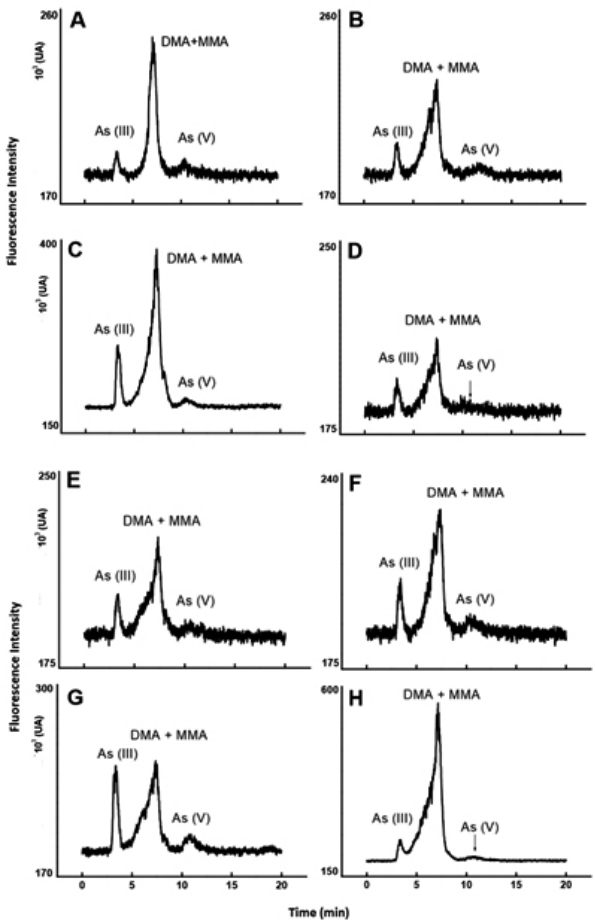

Figure 2. Determination of native As(III), DMA, MMA and As(V) in human urine samples.

Table 5. As(III) distribution in epithelial cell cultures of mice (n=6)

As(III)	1 nM	10 nM	100 nM
Intracellular	0.4 ± 0.1	8.0 ± 0.3	89 ± 5
Extracellular	0.6 ± 0.1	2.0 ± 0.1	11 ± 5

As(III), CTGF expression and myofibroblast-like phenotype induction in M-1 cells.

Evidence suggests that in pathologic conditions, tubule epithelial cells can undergo epithelial-mesenchymal transition (EMT), contributing to renal fibrosis[24]. Several factors are involved in this process, such as connecting tissue growth factor (CTGF). CTGF protein levels were evaluated using immunoblotting techniques. Figure 3 shows that CTGF protein expression showed a slight but non-significant increase after 16 h of As (III) treatment (Fold change: Control: 0.73 ± 0.08, 1 nM: 0.8 ± 0.2, 10 nM: 0 7 ± 0.2, 100 nM: 0.7 ± 0.1; p= non-significant). Starting from the premise that exposure to arsenic causes inflammation and that this leads to an increase of CTGF, we expected an increase in expression of CTGF when treated with 100 nM of As (III), as suggested by others. CTGF expression increases during pathologic conditions and is associated with EMT and becoming fibroblasts, which produces extracellular matrices and contributes to renal fibrosis [25]. Consequently, we looked for EMT cell phenotypes in M-1 cells.

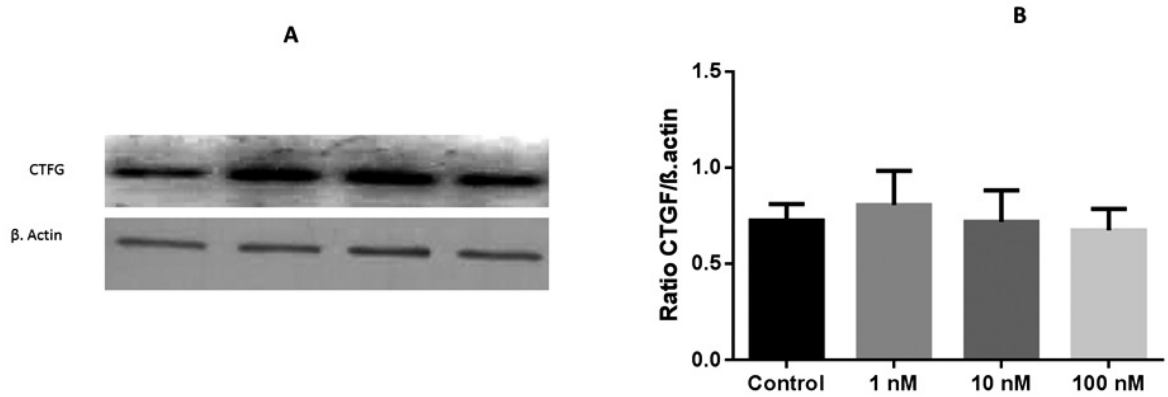

Figure 3. A. Representative immunoblotting showing connecting tissue growth factor (CTGF), a marker for fibrosis and β actin as a loading control. **B.** Quantification of 6 independent experiments showing CTGF / β actin ratio. n = 6.

To evaluate whether As (III) treatment induced EMT, M-1 cells were treated with As (III) at 100 nM. After 16 h, the cells were fixed in methanol, stained with β-actin antibody, and immunofluorescence was used to visualize the cell shapes. Figure 4 shows three representative fields demonstrating that the number of myofibroblast-like cells was augmented after 16 h of As(III) treatment. M-1 cells lost their normal morphologies of renal epithelial cells characterized by cytoskeletons surrounding circular core forms, while the cells treated with As(III) showed elongated shapes and suggested EMT. To further confirm our observations, the morphologies were quantified by morphometric analyses using ImageJ software based on cell lengths, as described in the methods section.

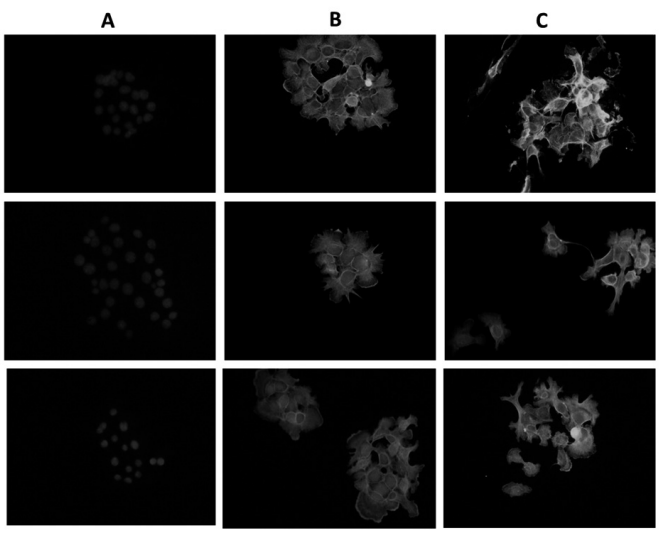

Figure 4. Representative images of M-1 renal collecting duct cell morphology using immunofluorescence of cells stained with β-actin antibodies as a cytoskeleton marker. **A.** Negative control without primary antibody but with secondary antibody **B**. Merged image showing nuclear staining with DAPI (blue) and β-actin (green) in the control cells. **C**. Merged image showing β-actin (green) and DAPI (blue) in cells treated with As (III) at 100 nM. As observed in the figure, after 16 h of As (III) treatment, the M-1 cells underwent EMT with typical myofibroblast-like phenotypes, as judged by their morphologies.

As shown in Figure 5, there was a significant increase in cell lengths after As (III) treatment (33 ±10 vs. 22±7 µm; P<0.05). Our results were consistent with previous studies conducted by Pereira´s group in 2007 [26]. In this study, Pereira et al, reported that As (III) caused a loss of endothelial monolayer integrity in HAECs (human aortic endothelial cells) exposed to 1.5 and 10 mM As (III) over 1, 6, 12 and 24 hour treatment times and described the formation of actin stress fibers [26]. Furthermore HL-60 cells (leukemia cells) exposed to As (III) over 48 hours showed morphological changes[27].

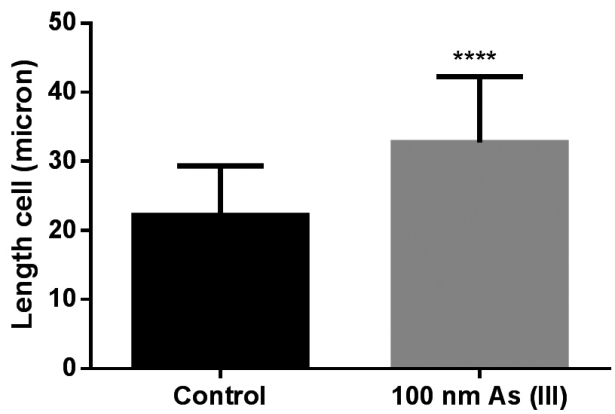

Figure 5. Morphometric analyses. Control (vehicle) and M-1 cells treated with 100 nM of As (III). n = 49 for controls; n = 28 for As (III). ****$P < 0.0001$

Our results showed that differences in CTGF expression could not be attributed to the EMT. This observation can be explained by the concentration of As(III) used and the time when CTGF was analyzed.

The minimum concentration of As_2O_3 for induction of apoptosis was 0.1 µmol L^{-1}. After 5 hours of incubation with 10 mmol L^{-1} of As(V) or As(III), significant cell death (hemolysis) occurred only in the As(V) treated cells[28]. Regarding to the incubation time, Sánchez-López et al. [29] established that increased CTGF expression in kidney cells was observed on the third day of incubation with Ang II and that the CTGF levels were associated with fibrosis after one week of Ang II infusion. Zhang et al. [30] established that there was an increase in mRNA expression when As (30 ppm) was given through tap water over 2 days. A study in Mexico demonstrated that 72 women (18-51 years) from different areas exposed to different concentrations of iAs in drinking

water resulted in As concentrations of 2-378 µg L^{-1}. Those subjected to high arsenic exposure had a significantly higher TGF-alpha concentration in BUC than those from areas with low arsenic exposure (128.8 vs. 64.4 pg m^{-1} g^{-1} protein, $p < 0.05$) [31]. This previous evidence suggests that extended incubation times may be not necessary to determine an increase in CTGF at different As(III) concentrations. On the other hand, the concentrations found in literature were not similar to those determined in this study[19].

CONCLUSIONS

Based on the evidence presented in this study, we found that workers occupationally exposed to mining and thermoelectric operations presented high levels of arsenic in urine, specifically As(III), which could be harmful for kidney cells. Our results suggest that urinary tract epithelial cells exposed to As(III) may result in epithelial-mesenchymal transition, which involves a functional loss of their tubular structures.

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