HEP-2 CELL LINE AS AN EXPERIMENTAL MODEL TO EVALUATE GENOTOXIC EFFECTS OF PENTAVALENT INORGANIC ARSENIC

HEP-2 COMO MODELO EXPERIMENTAL PARA EVALUAR LOS EFECTOS GENOTÓXICOS DEL ARSÉNICO INORGÁNICO PENTAVALENTE

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ABSTRACT

Early detection of toxic events induced by xenobiotics is necessary for a proper assessment of human risk after the exposure to those agents. The aim of this work was to evaluate the cell line HEp-2 as an experimental model to determine the genotoxic effects of sodium arsenate. To this end, we determined the metabolic activity cells by the MTT test on seven concentrations of arsenate that range from 27 to 135,000 μM, obtaining the median lethal concentration (LC_{50}), the lowest observed effect concentration (LOEC), and the not observed effect concentration (NOEC) of sodium arsenate at 24 h of exposition. According to the cytotoxic response obtained, we evaluated the genotoxic effect of the 27 and 270 μM concentrations by using the micronucleus assay and chromosomal aberrations test. We found a statistically significant increase ($p<0.05$) in the frequency of micronuclei between control cultures and those exposed to the highest concentration of sodium arsenate. Furthermore, the frequencies of nucleoplasmic bridges and tripolar mitosis were significantly higher in cell cultures exposed to the above concentrations compared to the control cultures ($p<0.05$). The participation of the glutathione system as response to the arsenate exposition was also analyzed, and a statistically significant increase in the glutathione content was found in those cells exposed to 27 μM of arsenate. The Glutathione S-transferase activity did not increase in the exposed cells compared to control cells, suggesting that the arsenate reduction involved other metabolic pathways in the HEp-2 cells. These results confirm that, under the conditions carried out in this study, sodium arsenate is genotoxic for HEp-2 cells. Therefore, we suggest that this cell line would be a good model for the assessment of the cytotoxic and genotoxic effects of xenobiotics on human cells.

Key words: cytotoxicity, genotoxicity, Glutathione, HEp-2 cell line

RESUMEN

La detección temprana de eventos tóxicos inducidos por xenobióticos es necesaria para una adecuada evaluación del riesgo humano ante la exposición a dichos agentes. El objetivo de este trabajo fue evaluar a la línea celular HEp-2 como modelo experimental para determinar los efectos genotóxicos del arseniato de sodio. Para ello, se determinó la actividad metabólica de las células mediante el ensayo de MTT, en siete concentraciones de arseniato de sodio en el rango 27-135.000 μM, determinando la concentración letal media (LC_{50}), la menor concentración de efecto observado (LOEC) y la mayor concentración de efecto no observado (NOEC) de arseniato de sodio para una exposición de 24 h. Teniendo en cuenta los datos de citotoxicidad, se evaluó el efecto genotóxico a las concentraciones 27 y 270 μM por medio del ensayo de micronúcleos y aberraciones cromosómicas, encontrando un aumento estadísticamente significativo en la frecuencia de micronúcleos entre el control y la mayor concentración arseniato de sodio ensayada. Además, la presencia de puentes nucleoplasmáticos y mitosis tripolar fue significativamente mayor en ambas concentraciones estudiadas con respecto al control. Se analizó la participación del sistema de glutatión como respuesta a la exposición al arseniato, encontrándose un aumento estadísticamente significativo en el contenido de glutatión en la concentración de arseniato de 27 μM. La actividad de la glutatión S-transferasa no aumentó, lo que sugiere que la reducción del arseniato implicó otra vía metabólica en las células HEp-2. Estos resultados confirman que el arseniato de sodio induce genotoxicidad en células HEp-2 en las condiciones realizadas en este estudio y por lo tanto este tipo de línea celular es un buen modelo para ensayos de citotoxicidad y genotoxicidad en los cuales se quiere evaluar el riesgo humano.

Palabras clave: Citotoxicidad, Genotoxicidad, Glutatión, HEp - 2.

Article 2 - research

INTRODUCTION

The genotoxic response of the biological system after the exposition to chemical agents depends on the capacity of metabolization, metabolic rate, repair enzymes activity and antioxidant response (Westerink et al., 2001; Wilkening et al., 2003; Raisuddin and Jha, 2004). Historically, researchers have employed different experimental designs, analytical techniques and biological models to study the genotoxic potential of certain compounds. Primary cultures or established cell lines of vertebrates are now commonly used to analyze the genotoxicity potential of drugs and complex mixtures. The use of in vitro models is promising because it reduces the use of experimental animals. An additional advantage of these models is that multiple tests can be performed with a relatively small amount of sample. The human epidermoid laryngeal carcinoma (HEp-2) cell line would be an ideal model for cytotoxic and genotoxic tests, due to its availability, stable phenotype, unlimited life-span, and the fact that it is easy to handle (Coalova et al., 2014). Few data exist on the use of HEp-2 cells to evaluate the genotoxicity of chemical agents, despite the fact that some studies were conducted with this cell line to evaluate the genotoxic effects of natural products and nanoparticles (Rizo et al., 2013; Osman et al., 2005; Andrighetti-Fröhner et al., 2006; Gomaa et al., 2015; Ahamed et al., 2015; Dos Santos Branco et al., 2015). Moreover, it is well-known that glutathione and glutatione-S transferases are involved in the metabolism of HEp-2 cells (Summer and Wiebel, 1981).

Among the agents studied in toxicology are the arsenical compounds, especially the inorganic arsenic species (iAs), which are frequently found as environmental contaminant. These chemical species may be either of geogenic and/or anthropogenic origin. Arsenic is a by-product of the copper, lead, zinc, tin and gold industry, as it is found as an impurity of many of these metals (Albiano and Villaamil Lepori, 2015). It is a metalloid that in contact with the humid air easily oxidizes to arsenic trioxide (arsenious anhydride) and, in contact with water, generates arsenate disodium. This toxic pentavalent is reduced to the trivalent form, and then to the organic forms that, in animals, will be excreted by urine. Acute intoxication causes, in the short term, respiratory tract irritation, and chronic intoxication produces multiparenchymal effects, involving the trachea and lung among other organs (Albiano and Villaamil Lepori 2015).

The cytotoxic and genotoxic effects of iAs, predominantly of the trivalent form (iAs^{III}), were widely reported, both in human epidemiology studies and in vitro and in vivo assays. These studies are based on the analysis of several end points analysis, such as micronuclei (MN), sister chromatid exchange (SCE), numerical and structural chromosome aberrations (CA), arrest of mitosis and apoptosis (Schuhmacher-Wolz et al., 2009; Basu et al., 2004; Yadav and Trivedi, 2009; Ahmed et al., 2011). Epidemiologic studies indicate that the human intake of iAs produces many effects including cancer (IARC, 2004; Environmental Protection Agency, 2001; European Chemicals Bureau, 2007). Some studies have revealed tumor development in mice (Tokar et al., 2010; Tokar et al., 2011; Waalkes et al., 2007) although different studies in animals models have given doubtful results (Huff et al., 2000; Schuhmacher-Wolz et al., 2009; Hughes et al., 2011).

Since there are evidences that iAs are not mutagenic in bacteria or mammalian cells (Rossman et al., 1980; Gebel, 2001), the genotoxicity could involve different mechanisms, such as the biomethylation of iAs (with subsequent hypomethylation of DNA), changes in the expression of genes of cell cycle control, DNA repair genes and oxidative stress due to the interaction with the glutathione system involved in the metabolization process (Kitchin, 2001; Miller et al., 2002; Rossman and Klein, 2011; Thompson, 1993).

Sodium arsenate, the pentavalent inorganic arsenic (iAs^V), is used as biocide in plague control and as a preservative of various industrial products (Figure 1A). It is the most predominant species of inorganic arsenic in the surface water, being a potential source of environmental contamination and human exposition. It is known that sodium arsenite (iAs^{III}) is more toxic than iAs^V, presumably because the cellular uptake of iAs^V is difficult due to their electric charge and competition with the cellular phosphate (Nakamuro and Sayato, 1987; Kochhar et al., 1996). In mammals, including humans, the iAs^V is partly reduced into the cells to As^{III} non-enzymatically by glutathione oxidation or enzymatically catalyzed by reductases and then methylated, forming monomethylated arsenicals (MMA) and dimethylated arsenicals (DMA) (Figure 1B). Differences in the reduction and methylation rates were found in biological models (Odanaka et al., 1980; Vahter, 2002). For example, a study showed that the rate of metabolic reduction of iAs^V was lower in HeLa cells than in HepG2 cells (Peel et al., 1991). Therefore, the iAs^V in HEp-2

cells could be reduced to iAs[III], by metabolic bioactivation involving the GSH/GST system, increasing thereby their genotoxic potential (Carmichael *et al.*, 1988). Considering the data registered for cytotoxicity and genotoxicity of iAs in other models and experimental designs, the aim of the present work was to apply short-term assays to analyze the HEp-2 cell line as an experimental model to determine the genotoxic effects of sodium arsenate (Na$_2$HAsO$_4$), the less toxic form of inorganic arsenic, in human cells.

A.
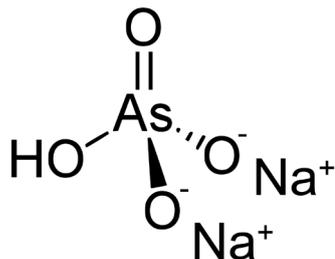
B.

Figure 1. Geometry of sodium arsenate formula (A), and reduction and methylation of As^V (B). Enzymes: PMP, purine nucleoside phosphorylase; AMT, arsenic methyltransferase; GST, GSH S-transferase. iAs: MMA^V, pentavalent monomethylarsonic acid; MMA^{III}, trivalent monomethylarsonic acid; DMA^V, pentavalent dimethylarsinic acid; and DMA^{III}, trivalente dimethylarsinic acid.

MATERIALS AND METHODS

Reagents

Modified Eagle's medium (MEM), MEM vitamin solution, MEM non-essential amino acid solution, and 0.05% tripsin-EDTA, were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from BIO-NOS (Buenos Aires, Argentina). The 1-chloro-2,4-dinitrobenzene (CDNB) (CAS N° 97-00-7), 3 - (4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (CAS N° 298-93-1), 4′,6-diamidino-2-phenylindole (DAPI) (CAS N° 28718-90-3), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (CAS N° 69-78-3), cytochalasin B (CAS N°

14930-96-2), glutathione (CAS N° 70-18-8), and sodium arsenate dibasic heptahydrate (CAS N° 10048-95-0) were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Giemsa (CAS N° 51811-82-6) was purchased from Biopur S.R.L. (Riccheri 195 Rosario, Argentina).

Cell culture

The human cell line HEp-2 was obtained from the ABAC (*Asociación Banco Argentino de Células*, Ciudad Autónoma de Buenos Aires, Argentina) and it was cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin and 2.5 μg/ml amphotericin B. Cells were cultured at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air. Cell culture medium was renewed twice a week. After 7 days, cells became confluent and ready to use. For all experiments, confluent attached cells were removed from cell culture dishes with 0.25% sterile trypsin and diluted with MEM/10% FBS. For MTT assay cells were reincubated into 96-well plates (0.2 ml; 2 x10^4 cells/well), cytokinesis-block micronucleus (CBMN) assay was performed into 6-well plates (2 ml; 3.8 x 10^5 cells/well), whereas GST activity and GSH content assays were performing reincubating the cells into Petri dishes (8 ml; 7.5 x 10^6 cell/plates).

MTT assay

To determine the range of concentrations of arsenate that keep the metabolic activity of cells, we exposed cell cultures to concentrations of iAs^V ranging from 27 to 27,000 μM. We used the method of Mossman (1983), with minor modifications. Briefly, cells were exposed for 24 h in serum-free medium to different dilutions of iAs^V. Following incubation, treatment cell culture medium was removed; cells were washed with PBS and replaced with 1 mg/ml of sterilized MTT solution. This MTT solution was freshly prepared in MEM containing no FBS, since it has been shown that FBS can dose-dependently inhibit formazan crystals formation, with a 50% decrease in these crystals when media with 5-10% FBS is used (Talorete, 2007). The plates with added MTT solution were then placed in the 5% CO$_2$ incubator for 90 min at 37°C. MTT solution was removed and 200 μl of ethanol was added to each well to dissolve the blue formazan crystals. Optical density was measured at 570 nm with background subtraction at 655 nm, in a BIO-RAD Benchmark microplate reader (BIO-RAD Laboratories, Hercules, CA). Results were

expressed as percentage of control (100% cell metabolic activity). Each assay involved 12 wells per condition and was performed in triplicate.

Glutathione-S-transferase activity (GST) and Glutathione Equivalents Content (GSH)

For the determination of GST activity and GSH content, cells were grown at confluence in Petri dishes, washed twice with PBS, and then treated with 27 and 270 mM iAs^V for 24 h. Then, cells were harvested, sonicated, and determinations were carried out in 11,000 x g supernatants.

Glutathione-S-transferase (EC 1.11.1.9) activity was measured by the Habig technique (Habig *et al.*, 1976). Briefly, standard assay mixture contained the enzymatic sample, 100 mM GSH, and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, in 100 mM phosphate buffer (pH 6.5), to a final volume of 0.8 mL. After adding CDNB, the change in absorbance at 340 nm was followed for 120 s. One GST unit was defined as the amount of enzyme that catalyzes the formation of 1 mmol of GS-DNB per minute at 25°C. Results were expressed as percentage of control.

Glutathione levels were measured following the method of Anderson (1985), with modifications. Briefly, 100 μL supernatant from the 11,000 × g sample was acidified with 50 μL of 10% sulfosalicylic acid. After centrifugation at 8,000 × g for 10 min, supernatant (containing acid-soluble GSH) aliquots were mixed with 6 mM 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) in 0.143 M buffer sodium phosphate (pH 7.5), (containing 6.3 mM EDTA). Absorbance at 412 nm was measured after 30 min incubation at room temperature. GSH content was determined by standard curve generated with a known GSH amount as reference. Results were expressed as nmol thiols (GSH equivalents) per mg proteins and presented as percentage of control.

Cytokinesis-block micronucleus (CBMN) assay

According to the results obtained on metabolic activity for MTT test, the CBMN assay was performed by exposing cells at 27 and 270 μM of iAs^V. Following the method of Fenech (2007), the cells were sub-cultured on glass coverslips in 6-well plates. Twenty four hours after seeding, the medium was removed and cells were treated with iAs^V at 27 and 270 μM in serum-free medium for 24 h. Then, cells were washed with PBS and incubated with medium containing cytochalasin B (final concentration 4 μg/ml)

for 18 h. After these treatments, cells were washed with PBS and fixed with glacial acetic acid: methanol (1:3 v/v) for 10 min at room temperature. Then cells were washed twice with PBS, stained with Giemsa (10% p/v), washed again with PBS and after that, the slides were mounted.

Scoring of slides and data analysis

The slides were examined under a Leica DMLB light microscope (1000×). One thousand cells from the negative control and treated groups were examine to calculate the binucleate cells frequency (BN), the micronuclei frequency (MNi), and the nuclear division index (NDI). The chromosome aberrations frequency (CA) as abnormal division were quantified at the same time that the MNi.

% MNi= (N° of BN cells whit MNi / 1000 BN cells) x100

$NDI = (M_1 + 2M_2 + 3M_3 + 4M_4) / N$, where M_1–M_4 represent the number of cells with 1-4 nuclei and N is the total number of cells scored.

% CA= (N° of chromosomal aberration / 1000 cells) x 100

Statistical analysis

Each experiment was performed three times in different weeks. Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by Dunnet's test using significant levels of $p<0.05$. Normality and homogeneity of variances were tested with the Lilliefors and Barlett tests, respectively. The LC_{50} value was estimated by nonlinear regression sigmoidal dose–response method. Graph Pad Prism 4 software was used for all statistical analysis.

RESULTS

Metabolic activity cells

The HEp-2 cell line was assayed using MTT assay to examine the effect of 27 to 135,000 μM iAs^V (27; 270; 27,000; 54,000; 64,500; 80,600; 121,000; and 135,000 μM). The corresponding LC_{50}, LOEC (lowest observed effect concentration), and NOEC (no observed effect concentration) were determined at 24 h (Table 1 and Figure 2).

Glutathione content and glutathione S-transferase activity

The glutathione system could participate in the bioactivation enzymatic or non-enzymatic of As^V by

Table 1. Toxicity values for HEp-2 cell line exposed to sodium arsenate for 24 h.

Sodium Arsenate Na_2HArO_4	LC_{50} (μM)	LOEC (μM)	NOEC (μM)
	1575.3 (1034.4 - 2399)	268,8	---

LC_{50}; median lethal concentration. LOEC; lowest observed effect concentration. NOEC; not observed effect concentrations, statistically determined. LC_{50} was estimated by nonlinear regression sigmoidal dose-response (variable slope) method. Graph Pad Prism 6 software was used for all statistical analyses. LC_{50}, LOEC, and NOEC were presented as mean of three independent analyses.
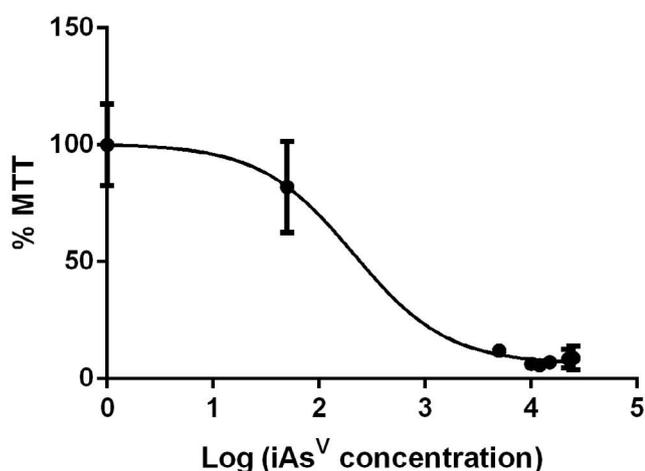

Figure 2. Dose-response curve for iAs^V effects fitted by non-linear regression. These effects were evaluated by the MTT test. Data are expressed as mean + SD (n = 8), relative to control cells (100% viability). MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation.

reduction to As^{III}. The results showed a statistically significant increase in GSH content in cells exposed to 27 μM iAs^V and a statistically significant decrease at 270 μM iAs^V, compared to the control (p<0.01and p<0.05, respectively) (Figure3A). GST activity did not show statistically significant differences (Figure 3B).

CBMN assay in the HEp-2 cell line
The CBMN assay was performed in the HEp-2 cell line to evaluate genotoxicity. Taking into account that the maximum concentration assayed for the genotoxicity test should not induce more than 50% of the cellular toxicity found with the MTT assay, the concentrations of iAsV selected for CBMN were 27 and 270 μM. The

NDI (Figure 4A), which ranges from 1.0 (cells have failed to divide) to 2.0 (all cells have divided once), was similar for the two concentrations tested, with no significant differences between the control (1.27), 27 μM (1.35) and 270 μM (1.15). A significant difference was found in the frequency of micronuclei, between control and 270 μM iAs^V (p<0.05) (Figure 4B and Figure 5A). The occurrence of nucleoplasmic bridges and tripolar mitosis were significantly higher in both concentrations respect to the control (Figure 4C and 4D; Figure 5B, 5C and 5D).

DISCUSSION

The iAs^V is present in nature, and its origin can be either geogenic or anthropogenic, in the latter as a result of industrial wastes. Moreover, iAs^V is the most predominant species of inorganic arsenic in the surface water, where the oxygen level is high enough to avoid the reduction to iAs^{III}. The reduction of iAs^V to iAs^{III} within the cell could involve the participation of GSH and GST, both of which are present in HEp-2 cells (Summer and Wiebel, 1981). However, our results showed an increase of the GSH level without increasing GST activity by exposing cells to different concentrations of iAs^V. These data indicate that GST is not involved in metabolization of iAs^V, and that the increase of GSH due to iAs^V exposition could be attributed to non–enzymatic reduction to iAs^{III}. Moreover, the GSH increase found in HEp-2 cells treated with iAs^V could be related to an induction of Γ–glutamylcysteine synthetase– the enzyme that controls the biosynthesis of GSH–due to the redox imbalance produced by iAs^V. Furthermore, GSH content decreased below control levels at higher concentrations of arsenic as a result of GSH consumption by reduction iAs^V to iAs^{III}.
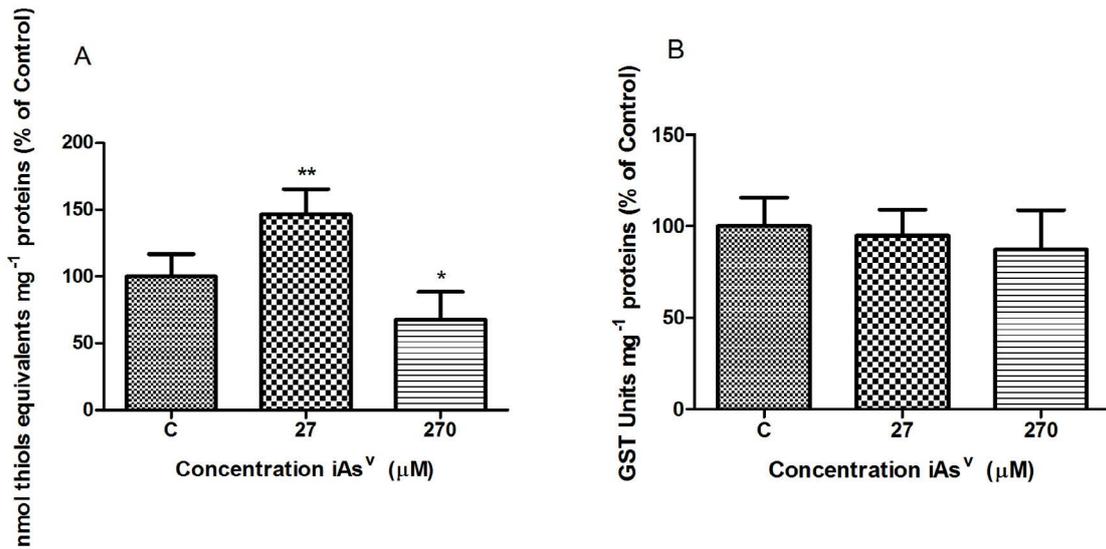
Figure 3. Glutathione Equivalents Content (A) and Glutathione-S-transferase activity (B). HEp-2 cells were exposed to iAs^V at different concentrations (27, and 270 µM) for 24 h. Results are reported as mean ± S.D. Significant differences between treatments and control are indicated by $*p < 0.05$ or $**p < 0.01$.
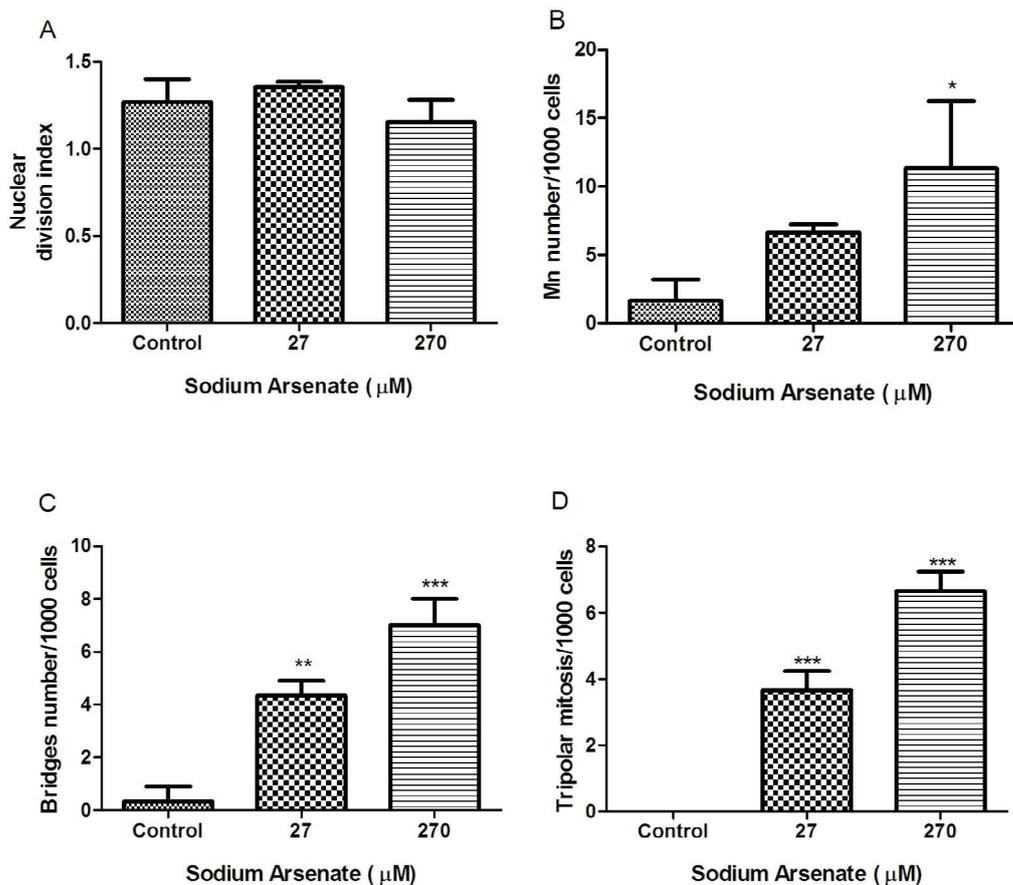

Figure 4. CBMN assay performed in HEp-2 cell line showing (A) nuclear division index, (B) number of micronuclei per 1,000 binucleated cells, (C) number of tripolar mitosis per 1,000 binucleated cells, and (D) number of nucleoplasmic bridges per 1,000 binucleated cells. Results were expressed as mean ± SD (n = 3). Significant differences between treatments and control are indicated by * ($***p < 0.0001$, $**p < 0.001$, and $*p < 0.05$).
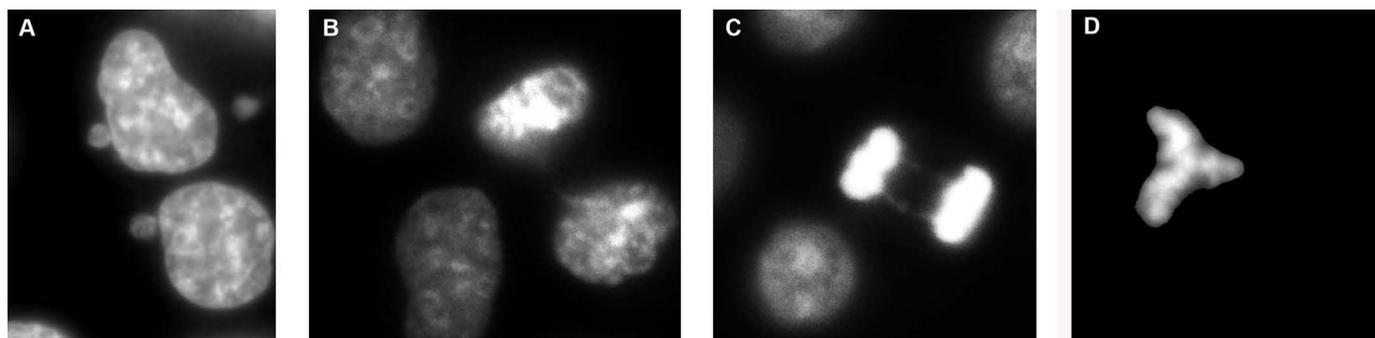
Figure 5. Nuclear morphology analysis of HEp-2 cell line. CBMN assay, an established biomarker for genomic instability, to evaluate susceptibility of HEp-2 cell line by measuring sodium arsenate-induced chromosomal damage endpoints (A, micronuclei; B,C nucleoplasmic bridges; and D, tripolar mitosis).
Nuclear DNA was stained with DAPI dye and visualized by fluorescence microscopy using filters for DAPI (λexc: 330-380 nm; λem: 435-485 nm).

The literature about the mutagenicity of iAs^{III} and iAs^V indicates that DNA is not the target of these chemical species (Gebel, 2001). In addition, an indirect genotoxic effect of iAs^{III} and iAs^V is involved due to their interaction with different molecules such as repair enzymes, cell cycle control proteins, apoptosis related gene products, nuclear lamins, defense cellular system against oxidative damage (GSH), metabolization enzymes, and tubulines of the mitotic spindle (Kirsch-Volders *et al.*, 2003). Studies on iAs^V genotoxicity show that at 24 h of exposition, iAs^V 1 μM is the lowest dose that induce MNi in CHO cells, whereas 16 μM and 10 μM were the lowest concentration of iAs^V that induced chromosomal aberrations in human umbilical cord fibroblasts and in human periferal lymphocytes, respectively (Dopp *et al.*, 2004; Florea *et al.*, 2005; Kligerman *et al.*, 2003; Oya-Ohta *et al.*, 1996).

The two concentrations of iAs^V tested in this study (27 and 270 μM) induced chromosomal abnormalities and MNi at 24 h after treatment, although only the highest concentration induced a significant statistically increase of MNi in HEp-2 cells. To analyze the genotoxicity of a chemical agent, the cytotoxicity should be very low or null for the concentrations tested (Gebel, 2001). Conservation of cell proliferation is necessary to manifest genotoxicity through MNi formation, since the MNi are formed during cellular division. In the present study concentrations inducing MNi do not affect cell proliferation in HEp-2 as indicated by the MNi formed by chromatin fragments or whole chromosomes, when the spindle attachment fails. Whereas the spindle failure was attributed to iAs^{III} exposition (Sciandrello *et al.*, 2002), the tripolar spindles would indicate its effects on microtubules and microtubule associated proteins (MAPs). This is in accordance with other studies (Liao *et al.*, 2007) and with the mechanism proposed by several authors to explain the toxicity of iAs^V, such as the depletion of ATP formation by replacement of phosphate during oxidative phosphorylation (Gebel, 2001). The genotoxic agents which exert their mode of action without binding to DNA show a threshold and a sublinear concentration–effect relationship (Elhajouji *et al.*, 1997). However, it is still unclear if the genotoxicity of iAs is characterized by this mode of action (Rudel *et al.*, 1996; Raja *et al.*, 2013). This is an interesting subject that could be included in future studies. There is currently a need for rapid and efficient delivery of results in *in vitro* systems that are actually predictive of the *in vivo* situation. This requires a versatile system in daily use, which shortens experimentation times. At the same time, the use of *in vitro* models reduces the number of animal experiments needed to address genotoxicity studies. According to our results, the biological model of HEp-2 cells seems to satisfy these criteria. In conclusion, under the conditions carried out in this study, the HEp-2 cell line allowed us to detect the cytotoxic and genotoxic effects of iAs^V. In addition, this model could be an alternative in a battery of assays of genotoxicity for the evaluation of human risk after the exposition to chemical substances, either of natural origin or associated with occupational risks.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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