Effect of subchronic exposure to inorganic arsenic on the structure and function of the intestinal epithelium

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ABSTRACT

Inorganic arsenic, the most toxic form of arsenic found in water and food, is considered a human carcinogen. Numerous studies show its systemic toxicity, describing pathologies associated with chronic exposure. The main pathway of exposure to inorganic arsenic is oral, but many of the events that occur during its passage through the gastrointestinal tract are unknown.

This study evaluates the effect of subchronic exposure to inorganic arsenic [As(III): 0.025–0.1 mg/L; As(V): 0.25–1 mg/L, up to 21 days] on the intestinal epithelium, using Caco-2 cells as an *in vitro* model. Inorganic arsenic produces a pro-inflammatory response throughout the exposure time, with an increase in IL-8 release (up to 488%). It also causes changes in the program of cell proliferation and differentiation, which leads to impairment of the cell repair process. In addition, subchronic exposure affects the epithelial structure, causing loss of microvilli, fundamental structures in the processes of intestinal absorption and digestion. Moreover, the exposure affects the epithelial barrier function, evidenced by an increase of Lucifer Yellow transport (103 – 199%). Therefore, it can be concluded that subchronic exposure to inorganic arsenic can alter intestinal homeostasis, affecting the mucosal layer, which performs the most important functions of the intestinal wall.

Keywords: Inorganic arsenic; intestinal epithelium; subchronic exposure; inflammation; permeability; wound healing; differentiation; proliferation; Caco-2 cells.

1. INTRODUCTION

Arsenic (As) is a metalloid that is widely distributed in the environment. About 200 million people are affected by consumption of water contaminated with levels of inorganic As that exceed the maximum limits recommended by the World Health Organization (10 µg/L; WHO, 2012). Food is also an important source of exposure to inorganic As. Seaweed is the food with the greatest concentrations of this form of As, which can reach 140 mg/kg in *Hizikia fusiforme* brown seaweed (Almela et al., 2002). However, cereals, especially rice and rice products, are the foods that make the greatest contribution to intake of inorganic As because consumption of them is so high (EFSA, 2009). Moreover, in areas with chronic arsenicism, cooking with contaminated water can also cause substantial increases in levels of this metalloid in food (Díaz et al., 2015).

Arsenic has been classified by the Agency for Toxic Substances and Disease Registry as one of the 20 most dangerous substances present in the environment (ATSDR, 2000). Furthermore, the inorganic form of As is considered a carcinogenic agent for humans (IARC, 2004). Various target organs of inorganic As have been described: liver, kidney, bladder, skin, and central nervous system. Numerous *in vitro* and *in vivo* studies have been conducted on these systems, which have made it possible for knowledge about the toxicity of this metalloid to advance. On the other hand, there are few studies on its toxicity on a gastrointestinal level. However, the digestive system is the gateway through which As enters the blood circulation, where it exerts its systemic toxicity; moreover, the digestive system is in continuous contact with the contaminant, especially in cases of chronic exposure.

In populations chronically exposed to inorganic As through drinking water, symptoms of dyspepsia, gastroenteritis and chronic diarrhea have been reported (Borgoño et al., 1977; Guha Mazumder and Dasgupta, 2011). In rodents chronically exposed to high concentrations of monomethylarsonic acid [104 weeks; MMA(V) 10–1000 mg/L], a metabolite of inorganic As, it has been shown that the large intestine is the target organ (Arnold et al., 2003), and there have been observations of enlargements of the intestinal wall, edemas, hemorrhages and necrosis, ulcerations or perforations of the mucosa, as well as a significant increase in the incidence of squamous metaplasia of absorptive epithelial cells of the colon and rectum. *In vitro* studies have shown that acute exposure to trivalent forms of As generates a pro-inflammatory response (increase of cytokines IL-6, IL-8, and TNFα) and produces oxidative stress (Calatayud et al., 2013, 2014, 2015) in human colonic epithelial cells.

All the data reported in the studies cited indicate that inorganic As can have a toxic effect on a gastrointestinal level. The effects on the digestive system can affect important functions such as absorption or digestion of nutrients, and may also cause loss of the intestine's barrier function, which is necessary to avoid the passage of undesirable substances and microorganisms into the systemic circulation. The toxic effects on intestinal cells that have been demonstrated in acute exposures may be exacerbated in chronic exposures, even at lower concentrations. That is why it is necessary to evaluate

the intestinal toxicity of As in these situations, which are frequent in many parts of the world, and also to determine what mechanisms are responsible for it.

The main aim of this study is to determine the alterations produced in the intestinal epithelium by continuous exposure to inorganic As, using Caco-2 cells as a cell model.

2. MATERIALS AND METHODS

2.1. Standard solutions of arsenic. The As(V) standard solution (1000 mg/L) was acquired from Merck (VWR). The standard of As(III) (1000 mg/L) was prepared by dissolving 1.320 g of As_2O_3 (Riedel de Haën) in 25 mL of 20% (m/v) KOH (Panreac), neutralizing with 20% (v/v) H_2SO_4 (Merck), and diluting to 1 L with 1% (v/v) H_2SO_4.

2.2. Conditions of cell culture and treatments with arsenic. The human colon carcinoma Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, number 86010202). The cells were maintained in 75 cm^2 flasks to which we added 10 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and 0.87 g/L glutamine. The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA), 1 mM sodium pyruvate, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.0025 mg/L of amphotericin B (DMEMc). During the maintenance, the cells were incubated at 37 °C in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. The medium was changed every 2–3 days.

When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 g/L) and EDTA (ethylene diamine tetraacetic acid, 0.22 g/L) and seeded in different supports for As exposure. For these treatments, we used DMEM supplemented as described above except for FBS. The quantity of FBS and the concentrations of As(III) and As(V) to be used were determined by evaluating two parameters: cell viability, by analyzing the reduction of resazurin sodium salt (Sigma), following the protocol described by Rocha et al. (2011), and the monolayer integrity, by microscopic visualization. After establishing the conditions for the study we evaluated the toxicological parameters that are detailed below (sections 2.3–2.8). In all the cases, the cells were treated with As(III) or As(V) from the moment of seeding and the media was replaced every 2 days by fresh As-spiked media.

The assays were performed with cultures between passages 20 and 35. All the reagents used were obtained from HyClone Laboratories.

2.3. Pro-inflammatory response. The cells were seeded in 6-well plates at a density of 2.6×10^4 cells/cm^2, and they were exposed to As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS. At the times stipulated (7, 14, and 21 days), the media were recovered for analysis of the pro-inflammatory cytokine IL-8, using the ELISA Human IL-8/CXCL8 kit (Sigma), following the manufacturer's instructions.

2.4. Cell proliferation. Cell proliferation was evaluated by determining the number of cells at each stage of the cell cycle by analyzing the quantity of DNA. For this purpose, 6-well plates were seeded at a density of 2.6×10^4 cells/cm^2, and they were exposed to As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS from the moment of seeding. The cells were analyzed at 2, 5, and 7 days post-seeding. After the treatment the cells were washed with PBS and recovered with trypsin/EDTA. The cell cycle was evaluated using the BD Cycletest™ Plus DNA kit (BD Bioscience), following the manufacturer's instructions. The samples were analyzed by flow cytometry (Beckman Coulter Epics XL-MCL).

2.5. Cell differentiation. The progress of intestinal cell differentiation was evaluated by analyzing gene expression of intestinal epithelium brush border proteins [isomaltase (*SI*), dipeptidyl peptidase 4 (*DPP4*), and villin 1 (*VIL1*)], transcriptionally up-regulated during enterocyte differentiation (Devriese et al., 2017) and of *MYC*, whose down-regulation is considered one of the essential mechanisms that allows the exit from the cell cycle and the onset of differentiation of Caco-2 cells (Leoni et al., 2012).

The cells were seeded in 6-well plates at a density of 2.6×10^4 cells/cm^2, and they were treated with As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS for 5, 7, and 14 days. After the exposure time the cell monolayer was washed with PBS and the cells were recovered with trypsin/EDTA for extraction of RNA using a NucleoSpin RNA II kit (Macherey-Nagel). The extracted RNA was quantified spectroscopically using a NanoDrop ND-1000 system (NanoDrop Technologies). First-strand complementary DNA (cDNA) was obtained from 200 ng of total RNA using a reverse transcriptase core kit (Eurogentec Headquarters).

qPCR was performed using the LightCycler® 480 Real-Time PCR system (Roche Diagnostics). Reactions were carried out in a final volume of 10 µL containing 5 µL LightCycler® 480 SYBR Green I Master Mix (2X, Roche), 2.5 µL cDNA (20 ng/µL), 1 µL of each forward and reverse primer (10 µM; Biolegio) (Table 1), and nuclease-free water. No-template controls were run to verify the absence of genomic DNA. As reference gene *RN18S* was employed. PCR efficiency curves for each gene were calculated using triplicates of a 10-fold dilution curve.

The qPCR conditions were 95 °C for 5 min, followed by 40 cycles: 10 s denaturation at 95 °C, 10 s annealing at 55 °C, and 20 s elongation at 72 °C. The melting curve of each sample was analyzed after each PCR run to confirm PCR product specificity. The data were analyzed with the Relative Expression Software Tool (REST 2009, QIAGEN).

2.6. Intestinal barrier function. To evaluate the intestinal barrier function, the paracellular permeability of Lucifer Yellow (LY) was analyzed. Cells were seeded at a density of 6.4×10^5 cells/cm^2 on a semipermeable polyester membrane (Transwell®, diameter 24 mm, pore size 0.4 µm, Corning) in 6-well plates. This insert allows separation of the well into two compartments: the upper or apical compartment, which *in vivo* would correspond to the intestinal lumen, and the lower or basolateral compartment, which would correspond to the interstitial space in contact with the blood capillaries. From the start the cells were exposed to As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS. The As solutions were added to the apical side (1.5 mL), and DMEMc with 7.5% FBS without As (2 mL) was added to the basolateral side.

LY transport was evaluated 7, 14, and 21 days after seeding. The marker was added at a concentration of 100 µM to the apical compartment of the control wells and the wells treated with As. The quantity of LY that reached the basolateral side after 1 hour of incubation was measured using a microplate reader (PolarSTAR OPTIMA reader, BMG-Labtech) at excitation/emission wavelengths of 485/520 nm.

2.7. Structure of the cell monolayer. This study was conducted by means of transmission electron microscopy (TEM). Cells were seeded at a density of 1.1×10^4 cells/cm^2 on 12-well plates with inserts (Transwell®, diameter 12 mm, pore size 0.4 µm, Corning), as described in section 2.6. From the start the cells were exposed to As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS in the apical compartment. After 7 or 14 days the monolayers were fixed on the inserts with a solution of 2.5% paraformaldehyde and 0.5% glutaraldehyde, which was added to both compartments (0.5 mL in the apical compartment and 1 mL in the basolateral compartment). After 15 minutes of incubation the fixative solution was withdrawn and the cells were washed 3 times with Sorensen buffer solution (0.1 M, pH 7.3), and they were kept in this saline solution until the moment of their preparation.

The fixed monolayers were incubated with a 1% solution of osmium tetroxide (London Resin Company Ltd) at room temperature for 2 h. Then immersions in increasing concentrations of ethanol (30, 50, 70, and 90%) were performed. After removal of the insert, the cells were embedded in LR white resin, and the polymerized resin was incubated in an oven at 60 °C. Then the monolayers were stained with lead citrate, and ultrathin cuts (60 to 90 nm) were made with diamond knives in an Ultracut UC6 Leica unit. The preparations were observed in a Jeol JEM-1010 transmission electron microscope.

2.8. Wound healing test. After exposure of the cells for 7 days to As(III) (0.025, 0.075, and 0.1 mg/L) and As(V) (0.25, 0.75, and 1.0 mg/L) in DMEMc with 7.5% FBS from the moment of seeding, the cells were recovered with trypsin/EDTA and seeded at a density of 8.5×10^4 cells/cm^2 on a culture-insert 2 well 24 (Ibidi), which consists of two reservoirs separated by a 500-µm-thick wall placed in the wells of a 24-well plate. After the cells had reached confluence, the inserts were gently removed, creating a gap of ~500 µm, the wells were filled with DMEMc with 7.5% FBS, and the migration was observed by microscope for 24 h.

2.9. Statistical analysis. The results were analyzed statistically by means of one-factor analysis of variance (ANOVA), with multiple post hoc comparisons, using the Tukey test (SigmaPlot version 13.0). Differences were considered significant when $p < 0.05$.

3. RESULTS

3.1. Optimization of assay conditions. Before performing the toxicity assays we determined the optimum conditions for conducting a 21-day continuous exposure study. Assays were considered valid if the cell viability was ≥75% and no destructuring of the cell monolayer was observed. Two variables were considered: FBS concentration (2.5%, 5%, and 7.5%) and As species concentration [As(III): 0.025, 0.075, 0.1, 0.5, 1 mg/L; As(V): 0.25, 0.75, 1, 3, 5 mg/L].

To begin the optimization, we used DMEM supplemented with 2.5% FBS, a relatively low concentration in order to avoid interference from the components of FBS due to the possible formation of complexes with the inorganic As. In this first assay we used the highest concentrations of As(III) (0.5 and 1 mg/L) and As(V) (3 and 5 mg/L), in which conditions we did not manage to maintain cell viability for more than 5 days (data not shown). The next assays were performed with higher concentrations of FBS (5% and 7.5%) and the As concentrations just specified. In this case the cultures remained viable for a longer time (7 days), but not long enough to carry out a subchronic exposure assay (data not shown). The last assay was performed with lower concentrations of As [As(III): 0.025, 0.075, and 0.1 mg/L; As(V): 0.25, 0.75, and 1 mg/L] and two concentrations of FBS (5% and 7.5%); only the cells with 7.5% FBS maintained viability and integrity for 21 days.

In light of the optimization assay data, the concentrations selected were: 0.025, 0.075, and 0.1 mg/L for As(III); 0.25, 0.75, and 1 mg/L for As(V). The As species solutions were prepared in DMEM supplemented with 7.5% FBS, 1 mM sodium pyruvate, 1% NEAA, 10 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.0025 mg/L amphotericin B.

3.2. Evaluation of pro-inflammatory response. Figure 1 shows the release of the pro-inflammatory cytokine IL-8 to the medium after 7, 14, and 21 days of exposure of cell cultures to various concentrations of As(III) and As(V) from the moment of seeding. The results indicate that the release of IL-8 was dependent on the As concentration employed, but not on the duration of the treatment.

The treatments with the highest concentrations of As(III) (0.075 and 0.1 mg/L) showed concentrations of IL-8 in the medium that were statistically higher than those of the control cells at all the times assayed. The increases in release of IL-8 were higher at the concentration of 0.075 mg/L (239–466%) than at that of 0.1 mg/L (166–335%). This result, which was found throughout the study, may have been due to the fact that the monolayers exposed to 0.1 mg/L had a lower response capacity because they were subjected to a situation of greater stress. In the case of As(V) we also observed a greater release of IL-8 at the highest concentrations (1.0 mg/L: 349–488%; 0.75 mg/L: 151–176%), demonstrating a dose-dependent immunotoxic effect. The cells treated with the highest concentration of As(V) still had a high response capacity, possibly because the exposure to As(V) did not produce a cell stress situation as accentuated as the treatment with As(III). It is worth noting that the quantity of cytokine released to the medium remained at higher levels than that of the control throughout the treatment with the highest concentrations of both forms of As [As(III) $\geq$ 0.075 mg/L; As(V) $\geq$ 0.75 mg/L]. These results indicate that the pro-inflammatory response of the intestinal cells subchronically exposed to inorganic As was maintained in time.

3.3. Effect on cell proliferation. The results obtained in this study show that after 2 and 5 days of exposure to As(III) and As(V) no differences were observed between the percentages of cells in the various cell cycle phases in relation to the control cells. The cell percentage ranges were as follows: 2 days (G0/G1 48–54%, S 23–26%, and G2/M 25–28%); 5 days (G0/G1 35–50%, S 25–36%, and G2/M 24–30%).

The cell percentages in the various phases after 7 days of exposure are shown in Figure 2. At this treatment time, differences were observed between the control cells and most of the treatments. In the cells exposed to inorganic As we observed higher cell percentages in the S and G2/M phases [control: 39%; As(III): 50–54%; As(V): 44–52%], whereas in the G0/G1 phases the cell percentage was higher in the control cells. These results suggest that the exposure to inorganic As for 7 days led to a larger number of cells being in the proliferative state (S + G2/M phases), and therefore a smaller number of cells had begun the process of differentiation.

3.4. Effect on cell differentiation. The process of differentiation in the intestinal epithelium cells takes place during their migration along the crypt axis from the base. This process, which is necessary for proper functioning of the intestine, can be studied by the use of Caco-2 cells as a model of the intestinal epithelium (Sambuy et al., 2005). In this kind of cell, differentiation occurs spontaneously and takes place when proliferation is inhibited by contact. As indicated in section 2.5, the expression of brush border proteins in the intestine increases during the differentiation process, whereas expression of the *MYC* gene, a Wnt target gene (Gregorieff and Clevers, 2005), decreases during the initial stages of the differentiation process. Table 2 shows the values of differential expression of these proteins in treated cells with respect to untreated cells after 5, 7, and 14 days of treatment.

The treatments with inorganic As affected expression of the differentiation markers assayed. All the As(III) concentrations reduced expression of the genes that encode *SI* and *VIL1*, although they did not affect expression of *DPP4*. This reduction took place in all the times for *SI* and after 14 days of exposure for *VIL1*. In contrast, *MYC* experienced an up-regulation that occurred after 5 days of exposure for the highest concentration of As(III).

The treatments with As(V) modified expression of the markers in a similar way to As(III), with *SI* and *MYC* being the genes most affected. The reduction in expression of *SI* took place after 5 days of exposure for all the concentrations, and the up-regulation of *MYC* occurred after 7 days of treatment. In view of the results we can conclude that, from the moment of seeding, the treatments with inorganic As led to an alteration in the process of intestinal cell differentiation.

3.5. Effect on the structure of the intestinal epithelium. The images of the ultrastructure of the Caco-2 cells after 7 and 14 days of contact with the arsenic species show substantial cytotoxic effects (Figure 3). Unlike the control cells, the cells treated with the highest concentrations of inorganic As [As(III) $\geq$ 0.075 mg/L; As(V) $\geq$ 0.75 mg/L] show areas without microvilli throughout the membrane, and in some areas where there are microvilli they present a certain degree of disorganization. In contrast, at the lowest concentrations of As(III) (0.025 mg/L) and As(V) (0.25 mg/L) these alterations of the epithelial structure did not appear. This indicates that inorganic As affects cell structures that are very important for the processes of absorption, digestion and permeability.

3.6. Effect on the barrier function of the intestinal epithelium. The barrier function is largely due to the protein complex formed by the tight junctions that seal the paracellular space. One way of evaluating this barrier function is by measuring paracellular transport of a fluorescent marker for which this is the main transport pathway. In this study we used Lucifer Yellow (LY) (Calatayud et al., 2011).

Figures 4 and 5 show transport of LY to the basolateral side in cells treated for 7, 14, and 21 days with As(III) and As(V), respectively. The highest concentrations of As(III) led to significant increases in LY transport with respect to the control cells for all the times assayed (103–199%), except in the cells treated with 0.075 mg/L As(III) for 21 days. The effect of As(V) was less noticeable and there was only a significant increase in LY transport in the monolayers treated for 14 days with the highest concentration of As(V) (130%). These data indicate that continuous treatment with the inorganic forms of As, especially As(III), increases paracellular transport and therefore produces looser intestinal monolayers. This leads to an increase in the permeability of the epithelium and a loss of its barrier function.

3.7. Wound healing assay. After injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the precise balance of migration, proliferation, and differentiation of the epithelial cells adjacent to the wounded area (Iizuka and Konno, 2011). These processes can be altered by As exposure, as we have already demonstrated. The results obtained after wounding Caco-2 cells exposed for 7 days indicated that the process of cellular repair was seriously impaired (Figure 6). Measurement of wound area after 24 h showed that control cells and cells treated with the lowest concentrations of As(III) and As(V) resealed the wounded area more quickly than cells treated with the highest concentrations of the two As species (Figure 7). This effect was seen as early as 24 hours post-wounding, where 94% of the wound was covered in control cells, compared with only 48% in cells treated with 0.1 mg/L As(III) and 50% in cells treated with 1.0 mg/L As(V).

4. DISCUSSION

Epidemiological studies have shown that inorganic As induces alterations of the immune system that vary depending on its concentration, the duration of the exposure, and the species of As (Di Gioacchino et al., 2007). There have been reports of phenomena of immune depression (Hernández-Castro et al., 2009) and immunotoxic effects derived from inflammatory processes (Islam et al., 2007). The immunotoxicity of this form of As has been studied in placenta, liver, and keratinocytes (Vega et al., 2001; Guha-Mazumder, 2005; Ahmed et al., 2011). Cytotoxic effects, particularly of As(III), have also been demonstrated in cells of the immune system (Hernández-Castro et al., 2009). However, few studies have explored the immunotoxic effects of the inorganic species on the intestinal epithelium (Calatayud et al., 2014, 2015), and they have only evaluated acute exposure to trivalent As.

On an intestinal level, it has been shown that cytokines that are considered pro-inflammatory may play a protective or inflammatory role, depending on the immunological state of the individual and the type or phase of the inflammatory process. This means that they may favor homeostasis and maintenance

of the intestinal barrier or generation of chronic intestinal inflammations (Bamias et al., 2012). However, when a chronic inflammatory process is established, not only do the cytokines no longer have the capacity to exercise their protective action but they also contribute to the damage to the intestinal epithelium. Calatayud et al. (2014, 2015) demonstrated an increase in pro-inflammatory cytokines (IL-6, IL-8, TNFα) in acute exposure to As(III), MMA(III), and DMA(III) in Caco-2 cells and in co-cultures of Caco-2 with peripheral blood mononuclear cells. This pro-inflammatory effect was also found in the present study, in which we observed an increase in release of cytokine IL-8 from the beginning of the subchronic exposure, which was maintained throughout the time of treatment with concentrations of As(III) ≥ 0.075 mg/L and of As(V) ≥ 0.75 mg/L (Figure 1). These data indicate that continuous exposure to inorganic As also produces an immunotoxic effect, with the additional problem that the effect is maintained throughout the exposure time. It is important to note that this immunotoxic effect appears with inorganic As concentrations that are common in the drinking water in countries such as Argentina, India, Mexico, and Bangladesh, and in foods such as rice, some kinds of bivalves, and seaweed (Almela et al., 2002; Carbonell-Barrachina et al., 2009; Rintala et al., 2014).

Many studies link the establishment of an inflammatory process to a loss of intestinal barrier function, which in turn favors the development of a chronic inflammatory process. It must be emphasized that the intestinal epithelium constitutes a layer with selective permeability that has the capacity to absorb substances necessary for the proper functioning of the organism and that prevents the passage of pathogens and toxic compounds. Therefore, the loss of this barrier leads to non-specific, deregulated intestinal permeability, with the result that harmful substances or microorganisms can come into contact with the immune system present in the lamina propria (Pastorelli et al., 2013). This contact with the intestinal immune system can induce an exacerbated immune response and the appearance of chronic processes. The loss of the barrier function described in inflammatory processes is partly due to the activity of pro-inflammatory cytokines on the tight junctions, the intercellular complex that maintains the structure and polarization of the epithelium (Capaldo and Nusrat, 2009). Specifically, Yu et al. (2013) demonstrated that cytokine IL-8 reduces expression of the tight junction proteins occludin, claudin-5, and zonula occludens-1, thus increasing the permeability of the epithelium.

The present study shows the negative effect of continuous exposure to inorganic As on the barrier function of the epithelial monolayer generated by Caco-2 cells. The treatments with As(III) and As(V) in which a pro-inflammatory response was observed also showed a greater transport of Lucifer Yellow (Figures 4 and 5), a compound that is mainly transported through the space between cells sealed by the tight junctions. In other words, continuous exposure to inorganic As leads to an increase in paracellular permeability and therefore a loss of the barrier function of the intestinal epithelium, possibly partly because of the underlying inflammatory process. This has only been demonstrated previously in acute exposure to high concentrations of As(III) (3 mg/L) and As(V) (8 mg/L) (unpublished data). Therefore,

this is the first study that indicates a loss of barrier function in continuous exposure at lower concentrations, which are representative of what happens in endemic areas or in population groups with a high consumption of certain food matrices.

As a result of continuous contact with these food contaminants and pathogenic agents and of the responses that they generate (oxidative stress, release of cytokines, etc.), the intestinal epithelium requires a continual process of renewal/repair to enable it to maintain its structure and function. In fact, it is considered the tissue with the most dynamic renewal process of all adult mammal tissues. The process of renewal of this epithelium depends on adequate progress of the proliferation of the stem cells present at the base of the crypt and on their differentiation as they make their way from the base to the tip of the villi, where they finally undergo a process of apoptosis and are eliminated into the lumen (van de Flier and Clevers, 2009). The studies on wound healing conducted in this work show that the process of repairing damaged tissue was impaired after continual treatment with inorganic As for only 7 days (Figures 6 and 7).

Numerous studies show that As can affect the rate of cell proliferation, increasing or decreasing it (Vega et al., 2001; Chowdhury et al., 2010). It has also been shown that exposure to As(III) affects certain signaling pathways in P19 mouse embryo cells, reducing cell differentiation (Bain et al., 2016). Moreover, the pro-inflammatory cytokines produced by exposure to As can also reduce the process of cell differentiation (Langen et al., 2001). The data obtained in the present study demonstrate that the cells treated with the highest concentrations of As(III) and As(V) showed a down-regulation of proteins of the brush border and an up-regulation of *MYC* (Table 2), so it can be concluded that they affected the differentiation process in intestinal epithelial cells. This is also reflected in the cell cycle; the cells treated with As presented a higher cell proliferation rate after 7 days of exposure (Figure 3) because there was a greater number of cells that had not entered the process of cell differentiation. Therefore we can say that the cells exposed continuously to As(III) (≥0.075 mg/L) and As(V) (≥0.75 mg/L) presented alterations in the processes of proliferation and differentiation, which may have caused the cell repair process to be altered at the highest concentrations. These alterations may have contributed to the loss of barrier function observed in the present study for the monolayers treated with the highest concentrations of As(III) and As(V).

Another noteworthy finding is the effect of inorganic As on the ultrastructure of the intestinal monolayer. In addition to affecting intercellular space, subchronic exposure to As(III) and As(V) reduces the microvilli of the epithelium. These microvilli increase the area of absorption of nutrients in the small intestine and the area of reabsorption of water in the large intestine. In addition to being involved in absorption processes, microvilli have digestive enzymes on their membrane that are responsible for digestion of disaccharides and polypeptides. Bennett et al. (2014) demonstrated that microvilli also play a part in defense against pathogens. According to that study, epithelial cells with a

reduction of microvilli interact with microbial particles to a greater extent than those that have intact microvilli. The authors link this to an electrostatic repulsion mechanism in the microvilli. Therefore, loss of these structures leads to a reduction of the absorptive, digestive, and defense capability of the intestinal cells.

In light of what has been said, we can conclude that continuous exposure to inorganic As at concentrations that are common in contaminated drinking water and some foods may affect the main functions of the intestinal epithelium (absorption, digestion, and defense) and also compromise epithelial repair mechanisms. All these toxic effects may be partly due to a pro-inflammatory process that is maintained during the time that the exposure lasts. The results of this *in vitro* investigation should be corroborated *in vivo* in order to confirm the gastrointestinal toxicity of inorganic As at low doses and long exposures.

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FIGURE CAPTIONS

Figure 1. Concentrations of IL-8 in the culture medium after exposure of Caco-2 cells to various concentrations of As(III) and As(V) for 7, 14, and 21 days. Values expressed as pg/10^6 cells (mean ± standard deviation; n=3). Asterisks indicate statistically significant differences with respect to the control ($p < 0.05$).

Figure 2. Cell percentages in the various phases of the cell cycle (G0/G1, S, and G2/M) in control cells and cells treated with various concentrations of As(III) and As(V) after 7 days of treatment. Values expressed as mean ± standard deviation (n=3). Asterisks indicate statistically significant differences with respect to the control ($p < 0.05$).

Figure 3. Transmission electron microscope image of Caco-2 cells: control (A); exposed to As(III) (0.1 mg/L; B) and As(V) (1 mg/L; C) for 7 days (A1, B1, and C1) and 14 days (A2, B2, and C2) on Transwell® systems.

Figure 4. Transport of Lucifer Yellow to the basolateral side in monolayers treated with As(III) (0.025, 0.075, and 0.1 mg/L) for 7, 14, and 21 days. Values expressed in μg/mL (mean ± standard deviation; n=3). Asterisks indicate statistically significant differences with respect to the control ($p < 0.05$).

Figure 5. Transport of Lucifer Yellow to the basolateral side in monolayers treated with As(V) (0.25, 0.75, and 1.0 mg/L) for 7, 14, and 21 days. Values expressed in μg/mL (mean ± standard deviation; n=3). Asterisks indicate statistically significant differences with respect to the control ($p < 0.05$).

Figure 6. Images of the wound healing assay in Caco-2 cells: control (A); exposed to As(III) (0.025 mg/L, B; 0.075 mg/L, C; 0.1 mg/L, D) and As(V) (0.25 mg/L, E; 0.75 mg/L, F; 1.0 mg/L, G) for 7 days. A1, B1, C1, D1, E1, F1, and G1 were obtained at time 0 and A2, B2, C2, D2, E2, F2, and G2 at 24 h.

Figure 7. Gap closure (% with respect to the initial wounded area) after 24 h in control cells and cells treated with various concentrations of As(III) and As(V) for 7 days. Asterisks indicate statistically significant differences with respect to the control ($p < 0.05$).

Table 1. Sequence and efficiency of the oligonucleotides used in the study of cell differentiation.

Gene	GenBank ID	Sequence 5'-3'	Amplicon (bp)	Efficiency
SI	*NM_001041*	F: AATCAGACACCCAATCGTTTCC R: GGGCAACCTTCACATCATACAA	134	2.12 ± 0.17
DPP4	NM_001935.3	F: GTGGCGTGTTCAAGTGTGG R: CAAGGTTGTCTTCTGGAGTTGG	111	2.01 ± 0.32
MYC	NM_002467.4	F: GTCAAGAGGCGAACACACAAC R: TTGGACGGACAGGATGTATGC	173	2.02 ± 0.07
VIL1	NM_007127.2	F: CTGAGCGCCCAAGTCAAAG R: AGCAGTCACCATCGAAGAAGC	127	2.04 ± 0.01
RN18S	NR_003286.2	F: CCATCCAATCGGTAGTAGCG R: GTAACCCGTTGAACCCCATT	151	2.00 ± 0.01

Table 2. Relative expression of differentiation markers (*SI*: isomaltase; *DPP4*: dipeptidyl peptidase 4; *VIL1*: villin 1; *MYC*) of Caco-2 cells treated with various concentrations of As(III) and As(V) for 5, 7, and 14 days. Values expressed in log base 2 (mean ± standard deviation, n=3). An asterisk indicates statistically significant down-regulation (red) or up-regulation (blue) with respect to the control ($p < 0.05$).

Days	Treatments (mg/L)	*SI*	*DPP4*	*VIL1*	*MYC*
5	As(III) 0.025	0.51 ± 0.07*	0.86 ± 0.16	0.96 ± 0.13	1.66 ± 0.23
	As(III) 0.075	0.22 ± 0.03*	0.90 ± 0.14	0.76 ± 0.13	2.14 ± 0.39
	As(III) 0.1	0.30 ± 0.05*	0.89 ± 0.13	0.85 ± 0.12	2.41 ± 0.36*
	As(V) 0.25	0.60 ± 0.09*	0.85 ± 014	1.04 ± 0.17	1.84 ± 0.28
	As(V) 0.75	0.14 ± 0.04*	0.56 ± 0.12	0.63 ± 0.12	2.23 ± 0.57
	As(V) 1.0	0.11 ± 0.02*	0.62 ± 0.13	0.56 ± 0.11	2.29 ± 0.39
7	As(III) 0.025	0.40 ± 0.08*	1.08 ± 0.21	1.34 ± 0.42	2.41 ± 1.41
	As(III) 0.075	0.10 ± 0.03*	1.20 ± 0.27	1.08 ± 0.31	10.80 ± 6.16*
	As(III) 0.1	0.09 ± 0.04*	1.50 ± 0.42	1.03 ± 0.34	23.83 ± 14.0*
	As(V) 0.25	0.33 ± 0.05	1.32 ± 0.19	1.06 ± 0.17	12.86 ± 6.97*
	As(V) 0.75	0.15 ± 0.05	1.22 ± 0.32	0.86 ± 0.29	17.02 ± 9.84*
	As(V) 1.0	0.11 ± 0.04*	1.75 ± 0.49	0.88 ± 0.29	18.53 ± 10.88*
14	As(III) 0.025	0.41 ± 0.06*	0.89 ± 0.11	0.72 ± 0.11*	1.74 ± 0.25*
	As(III) 0.075	0.005 ± 0.002*	1.06 ± 0.24	0.47 ± 0.11*	8.14 ± 1.85*
	As(III) 0.1	0.004 ± 0.001*	0.94 ± 0.24	0.41 ± 0.06*	7.50 ± 1.30*
	As(V) 0.25	0.15 ± 0.04*	0.58 ± 0.08*	0.62 ± 0.11	1.88 ± 0.31*
	As(V) 0.75	0.006 ± 0.001*	0.56 ± 0.10*	0.25 ± 0.04*	3.31 ± 0.64*
	As(V) 1.0	0.009 ± 0.002*	0.82 ± 0.23	0.32 ± 0.07*	5.12 ± 1.08*

Figure 1.

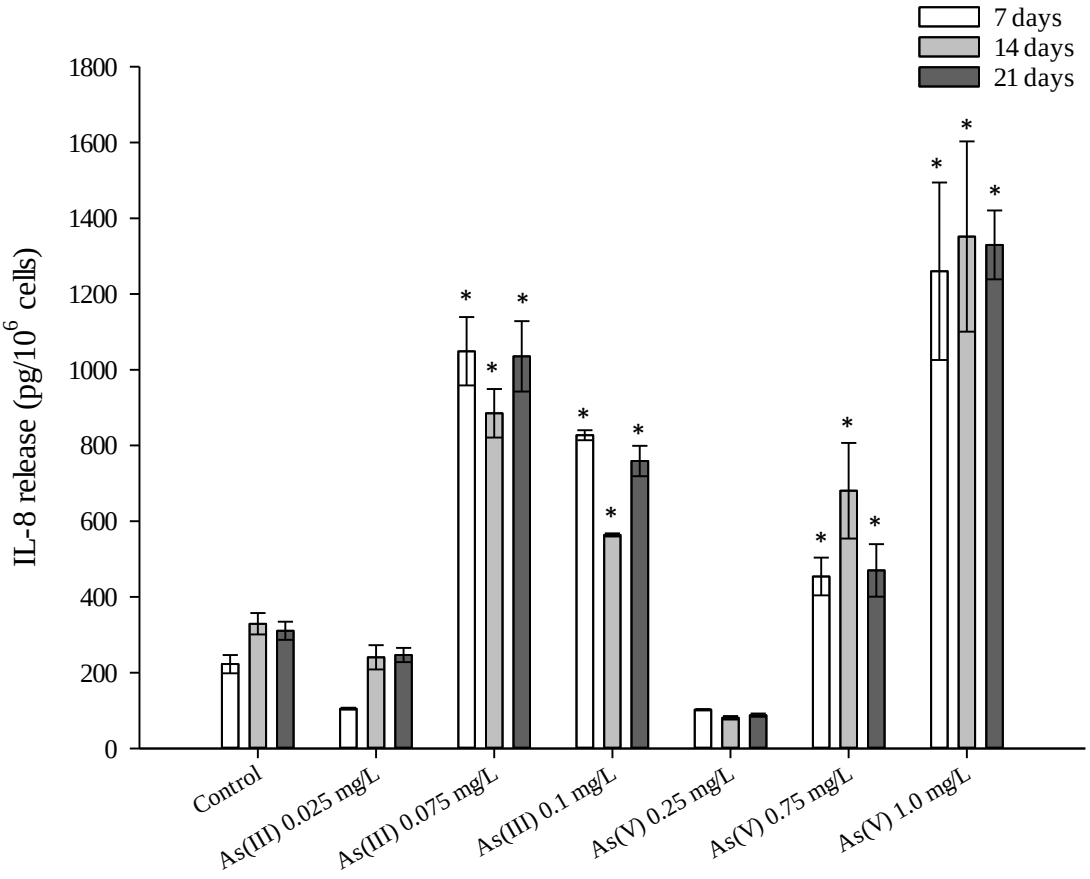

7 days
14 days
21 days
IL-8 release (pg/10^6 cells)
1800
1600
1400
1200
1000
800
600
400
200
0
Control
As(III) 0.025 mg/L
As(III) 0.075 mg/L
As(III) 0.1 mg/L
As(V) 0.25 mg/L
As(V) 0.75 mg/L
As(V) 1.0 mg/L

Figure 2.

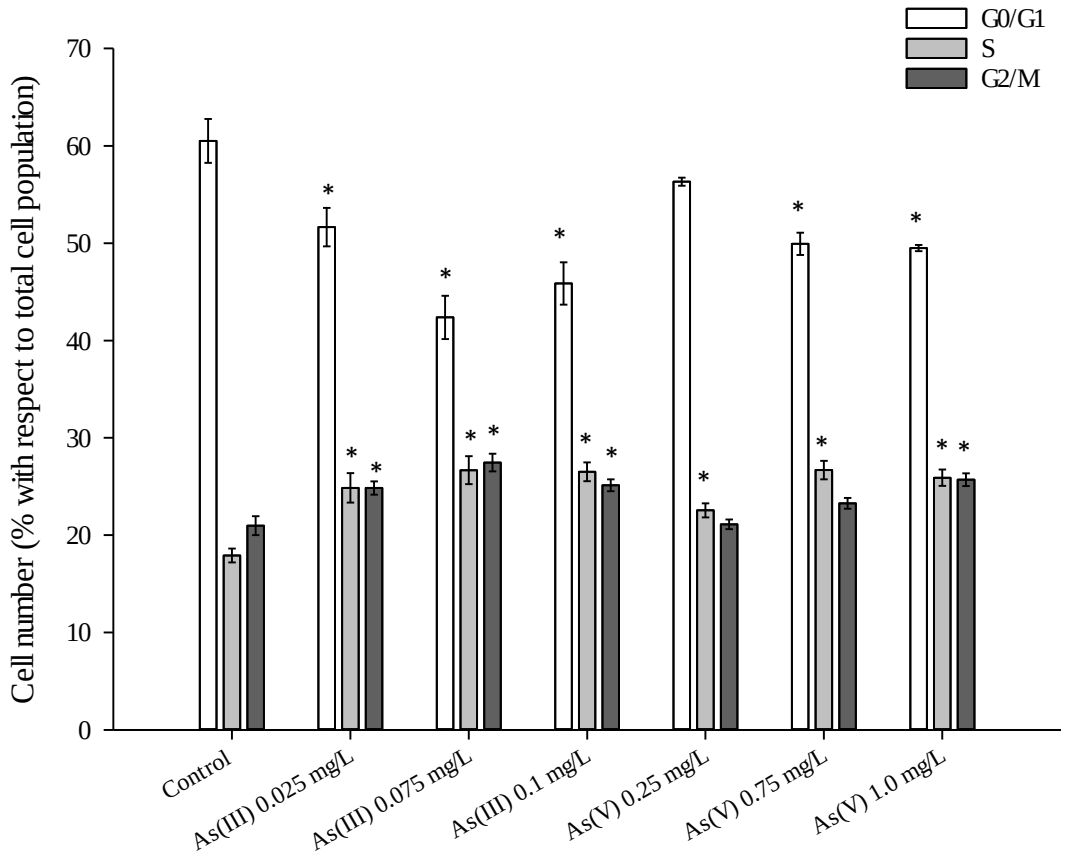

G0/G1
S
G2/M
Cell number (% with respect to total cell population)
70
60
50
40
30
20
10
0
Control
As(III) 0.025 mg/L
As(III) 0.075 mg/L
As(III) 0.1 mg/L
As(V) 0.25 mg/L
As(V) 0.75 mg/L
As(V) 1.0 mg/L

Figure 3.

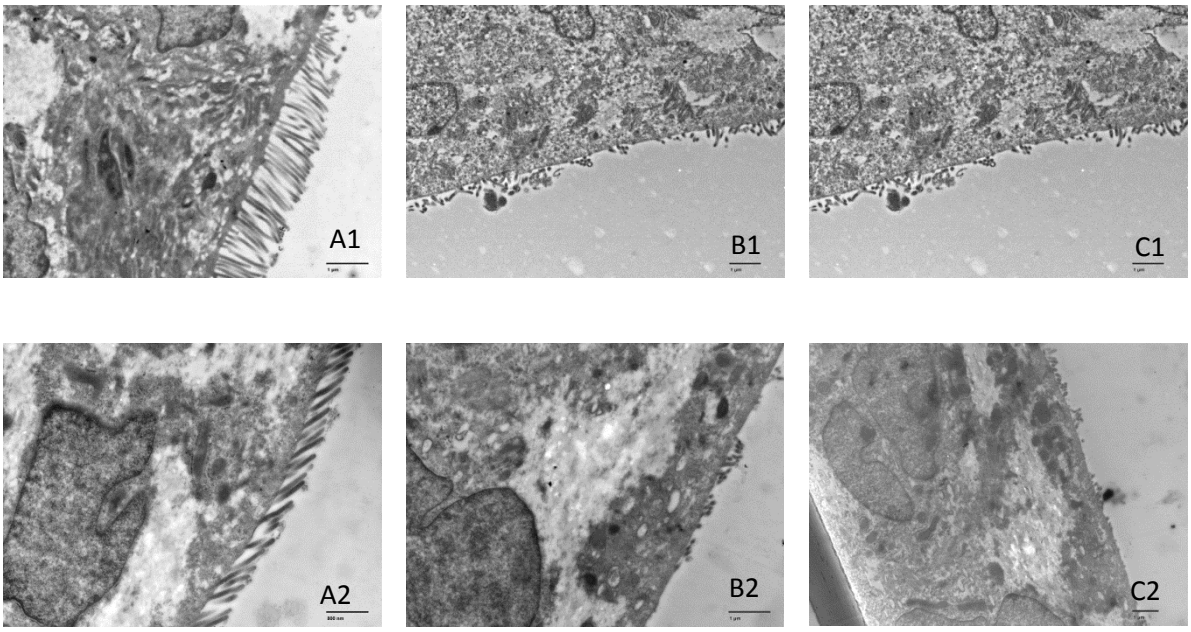


Figure 4.

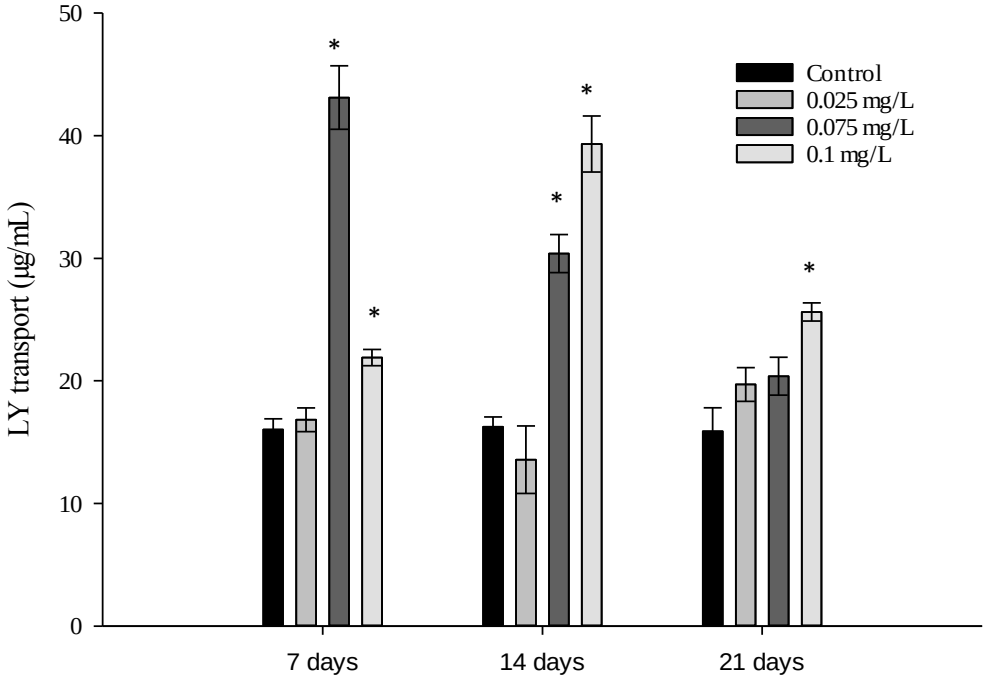

LY transport (μg/mL)
50
40
30
20
10
0
Control
0.025 mg/L
0.075 mg/L
0.1 mg/L
*
*
*
*
*
7 days
14 days
21 days

Figure 5.

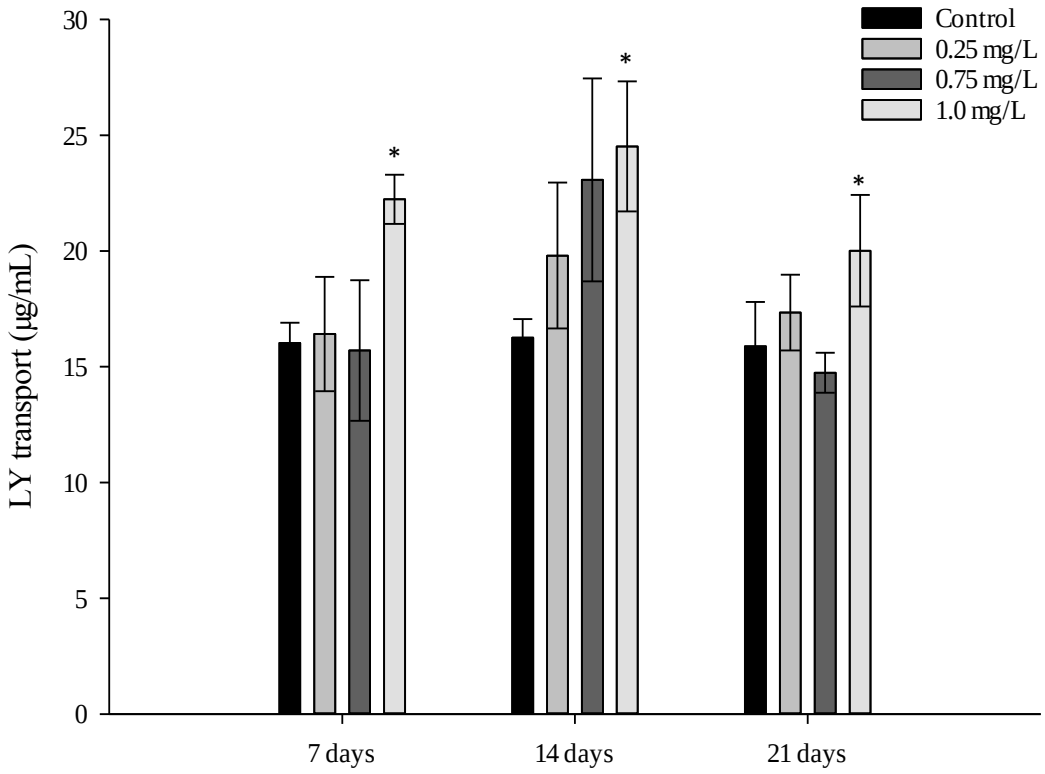

Control
0.25 mg/L
0.75 mg/L
1.0 mg/L
LY transport (µg/mL)
30
25
20
15
10
5
0
*
*
*
7 days
14 days
21 days

Figure 6.

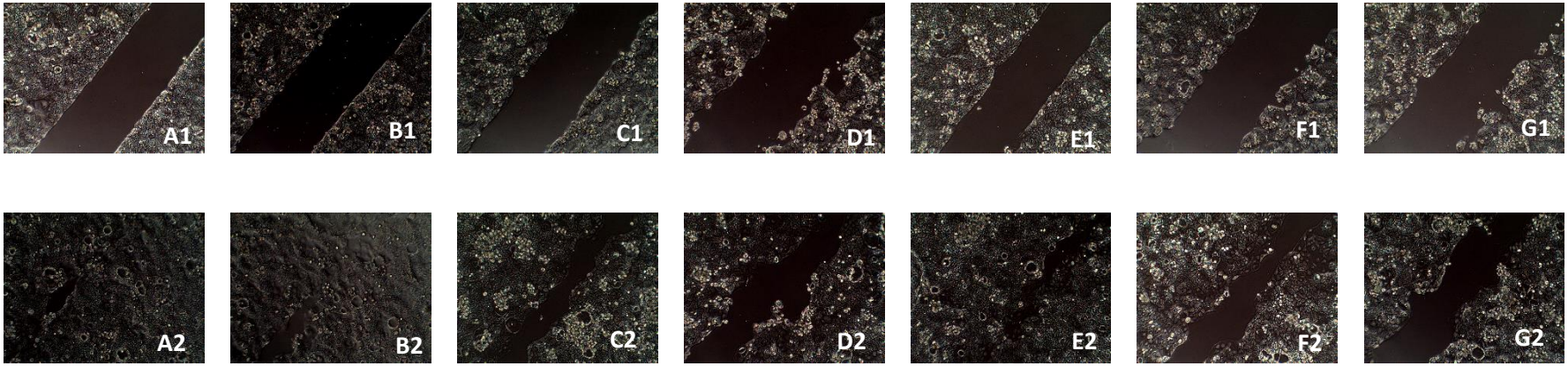
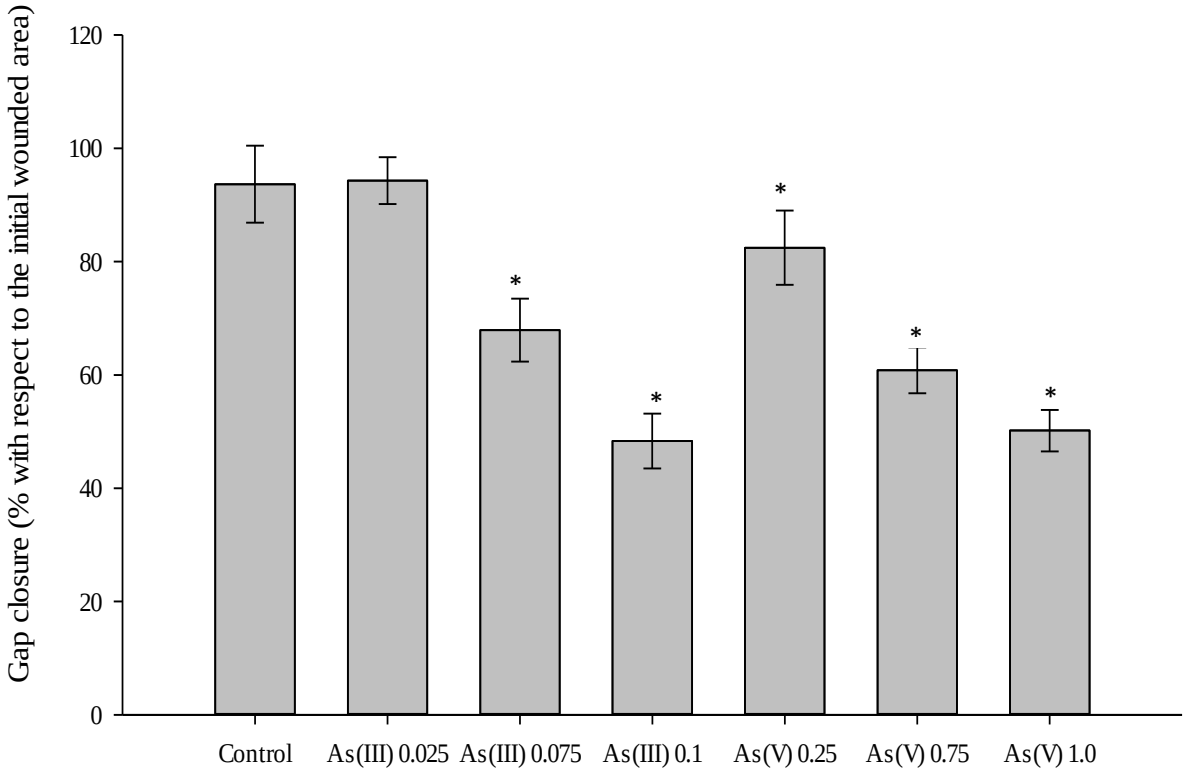


Figure 7.


Gap closure (% with respect to the initial wounded area)
120
100
80
60
40
20
0
*
*
*
*
*
Control
As(III) 0.025
As(III) 0.075
As(III) 0.1
As(V) 0.25
As(V) 0.75
As(V) 1.0