In Vitro Toxicity Assessment of Methylmercury, Arsenic (III and V) with a Cell-Based Model

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Posted Date: 4 December 2024

doi: 10.20944/preprints202412.0373.v1

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Article

In Vitro Toxicity Assessment of Methylmercury, Arsenic (III and V) with a Cell-Based Model

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Abstract: The negative health effects associated with exposure to heavy metals are of global concern, so their toxic mechanisms and potency should be identified to develop effective prevention. In this sense, we analysed the toxicity of arsenic (+3 and +5 valences) and meHg in intestinal epithelial cells (H4) and blood macrophages (TLT). We performed crystal violet, MTT, proliferation, ROS, H2ax, cell cycle arrest, apoptosis and cell shape assays with different concentrations of these elements to determine the potency and mechanism of their toxicity. The potency of the heavy metals differs in both cell types and among each element. In addition, all the selected heavy metals induced ROS formation and histone exposure, but each element had different pathways associated with their toxicity. For example, each element induced cell cycle arrest in different phases of H4 and TLT cells, and suggest that each element induce different cell death pathways in each cell type. Understanding the mechanisms of toxicity may help to implement effective strategies for the prevention or mitigation of the heavy metal toxicity.

Keywords: heavy metals; in vitro toxicology; arsenic; methylmercury; intestinal epithelial cells; blood macrophages; toxicity assessment

1. Introduction

Heavy metals are found in many regions of the world and there are various sources of exposure, in particular the environment and food [1,2]. Heavy metals have different forms that are strongly influenced depending on the source [3], and so is their toxicity, which depends on several factors (organic or inorganic form, or the valence, f.i.) [4]. This indicates the need to analyse the factors determining the spectrum of adverse health effects associated with exposure to these elements.

There are several ways to measure the toxicity of heavy metals, and subsequently, many studies have been conducted [5–9]. These toxicological studies were based on different models, *in vivo*, *in silico* and *in vitro*. We will focus on *in vitro* model since is the model that has the less paradoxical effects because the source of the model is taken from the target population (human cells) [10]. In a previous study, Caco-2 cell lines were used to assess the toxicity of different arsenic species [11]. In another study, different cell lines (rat and human hepatocytes, human epidermal keratinocytes and human bronchial epithelial cells) were used to evaluate the toxicity of different arsenic species [5]. The toxicity of methylmercury has also been investigated in other *in vitro* studies using different cell lines (HT-22 cell line, glioblastoma (U373) and neuroblastoma (B103)) [12,13]. These studies provide valuable information but considering the most common route of exposure of arsenic [1,2], toxicity studies should focus also on arsenic toxicity on intestinal epithelial and blood macrophages. And interestingly, research on the toxicity of these contaminants in intestinal epithelial cells is quite limited.

With this in mind, we evaluated the toxicity of arsenic (trioxide and pentoxide) and methylmercury with two different cell lines (intestinal epithelial cells and blood macrophages) in this manuscript. We performed proliferation test, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium

 doi:10.20944/preprints202412.0373.v1

bromide MTT assay, crystal violet and 2'-7'dichlorofluorescin diacetate (DCFH-DA) assay to determine the cytotoxicity, Annexin V/Vybrant/PI combined test to determine apoptotic cells (early and late) and cell morphology, and the H2ax for genotoxicity assessment of the selected compounds in both cell lines. We have observed that the toxicity of these elements varies across the tested compounds and cell types. The following chapters will detail these differences and their implications.

2. Materials and Methods

2.1. Cells

H4 cells are nontransformed epithelial cell line, derived from the small intestine of 20- to 22-wk gestation fetuses and characterized by Sanderson et al. [14]. Human blood macrophages called TLT (CVCL_6C16) were obtained at the University of Maribor (Slovenia) [15].

2.2. Cell Culture Medium

H4 and TLT Cells were cultured in DMEM Advanced (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5 wt% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA USA). Penicillin (100 U mL−1, Sigma), and streptomycin (1 mg*/mL−1, Fluka, Buchs, Switzerland) were also added to the cell culture medium. Cells were cultured in 25 cm2 culture flasks (Corning, New York, USA) at 37 °C and 5% CO_2.

2.3. Chemicals

The chemicals 3-(4,5-dimethylthiazole-2-yl)-2,5-phenyl tetrazolium bromide (MTT), sodium arsenate dibasic heptahydrate (*as V*), methylmercury(II) chloride (*meHg*) and 2'-7'dichlorofluorescin diacetate (DCFH-DA) were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Vybrant™ DyeCycle™ Green Stain (Vybrant), propidium iodide (PI) and sodium arsenite (*as III*) were purchased from Thermo Fisher Scientific (Waltham, MA USA). Phycoerythrin and a cyanine dye (PE-CY5) conjugated antibodies against Annexin V were purchased from (BD Biosciences, USA).

2.4. Method Overview

We treated the samples for 24 hours with *as III*, *as V* and *meHg* and analysed their toxicity with different *in vitro* tests (Figure 1), described below in the following subchapters.

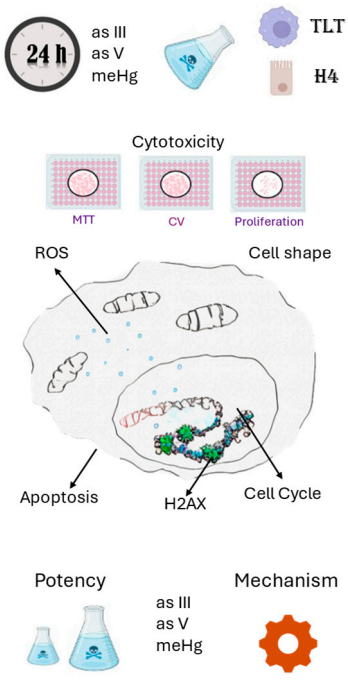

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Figure 1. The experimental setup for the analysis In vitro toxicity assessment of methylmercury, arsenic (III and V). TLT and H4 cells were incubated with different concentrations of *as III*, *as V* and *meHg*. We tested this concentration with different cytotoxicity methods and test for reactive oxygen species (ROS) production to determine the potency of the tested compounds in the selected cell types. We limit the concentrations for further tests (H2AX, cell cycle arrest, apoptosis and cell shape). We analyse the results gathered from all the mentioned tests and estimate the compounds potency and mechanism for toxicity.

2.5. Cytotoxic Assays

We cultured the cells till they formed a monolayer (100% confluency). We followed the protocol for MTT and crystal violet (CV) described by van Meerloo et al. [16] Peters et al. [17], and Kim and Hue [18], respectively. We measured the absorbance of the samples (570 nm and 590 nm for MTT and CV, respectively) with the VARIOSKAN (Thermo Fisher Scientific, Waltham, MA, USA). We followed the protocol for MTT as described in a previous work [19] (thus, we measured the absorbance at 570 nm) but we seeded substantially fewer number of cells (3,000, in comparison to the 20,000 cells in a monolayer) to observe the toxic effect in proliferating cells. In all three tests, we divided the absorbance of the treated samples with the absorbance of the untreated samples.

We tested various concentrations of *as III*, *as V* and *meHg* in Crystal Violet [1 - 2.5 – 5 – 10 – 25 – 50 - 100 mg/L]. The contaminant concentration in the MTT were in a narrower range [1 – 5 – 10 – 25 – 50 mg/L]. We treated the samples with the lowest contaminant concentrations when analysing the toxicity of contaminants in proliferation cells [0.25 – 0.5 – 1 – 2.5 – 5 – 10 mg/L]. In H4 cells, the range was determined to be [0.25 – 5 mg/L], while the TLT samples were treated within the range [0.5 – 10 mg/L].

2.6. Reactive Oxygen Species (ROS)

We cultured the cells till they formed a monolayer and determine the ROS production of the cells using the DCFH-da method [18]. We inoculated the cells with 50 µM of DCFH-da for an hour and measured the ROS production with the VARIOSKAN. We excited the DCFH at 485 nm and read the emission at 530 nm. We divided the relative fluorescence units (RFU) of the treated samples with the RFU measured in the untreated samples.

We tested various concentrations of *as III*, *as V* and *meHg* [1 – 5 – 10 – 25 – 50 - 100 mg/L].

2.7. H2AX

The assay consists of two directly conjugated antibodies: a phospho-specific ataxia telangiectasia mutated kinase (ATM) (Ser1981) conjugated to PE and a phospho-specific histone H2AX conjugated to PECy5 to measure activation of the DNA damage response. We followed the protocol previously described by Valverde and Sánchez-Brito [20] and measured with the Muse® Cell Analyzer (Luminex, Austin, TX, USA). We then determined the frequency of the presence of the signal from both antibodies (activated cells), the frequency of ATM positive cells (inactivated cells) and the frequency of double negative cells (non-expressing).

We adjusted the concentrations of *as III*, *as V*, and *meHg* based on the toxic effects observed in previous tests. Consequently, we treated the cells with the following concentration ranges according to the contaminants: *as III* [1 – 6 – 12 mg/L], *as V* [5 – 15 – 30 mg/L] and *meHg* [1 – 5 – 10 mg/L].

2.8. Apoptosis/Cell Cycle/Cell Shape

We collected cells and added 100 µL of an Annexin V PE-CY5 with binding buffer mixture (1:20) and incubate them for 15 minutes. We washed the cells with 400 µL PBS and centrifuge them 300g x 5minutes. After discarding the supernatant, we stained the cells with 50 µg/mL of PI and 5 µM of Vybrant and incubated them for additional 20 minutes. We excited the Annexin V PE-CY5, PI and Vybrant dyes with a laser at a wavelength of 488 nm and observed the emission in channel II (Vybrant), channel IV (PI) and channel XI (Annexin V PE-CY5). Additionally we observed the cell

 doi:10.20944/preprints202412.0373.v1

morphology in channel IX (brightfield) [21]. We considered the cells where the dye was present in channel IV as late apoptotic cells [6]. In the remaining cells, we determine the cells where the dye was present in channel XI as early apoptotic [22]. We determine the cell cycle considering the fluorescence intensity in channel II [23]. The cell shape was analysed with the IDEAS (Millipore) [24].

To continue with the H2ax analysis, we treated the cells with the same concentration ranges of the selected contaminants: *as III* ([1 – 6 – 12 mg/L]), *as V* ([5 – 15 – 30 mg/L]), and *meHg* ([1 – 5 – 10 mg/L]).

2.9. Statistical Analysis

The statistical analysis and graph designing was made with the R program. We used the ANOVA-tukey HSD test to compare the assessments of the CV, MTT, proliferation and ROS methods. We used Chi-square to test the difference between the frequencies of the treated and untreated cells in H2ax, Cell cycle, Apoptosis and Cell shape tests.

3. Results

3.1. Cytotoxic Assays

We performed the CV test to initially assess the toxicity of the chosen heavy metals on H4 and TLT cells. We observed significant cytotoxic effect on both cell types when exposing them to concentrations ranging from 10 to 25 mg/L of as III and meHg (Figure 2A). We only observed cytotoxicity on TLT cell when exposing them to 100 mg/L of as V, while we didn't observe any significant cytotoxicity effect on H4 cells. Noteworthy, there was a significant difference in the viability assessment between the H4 and TLT cells treated with meHg (Figure 2A).

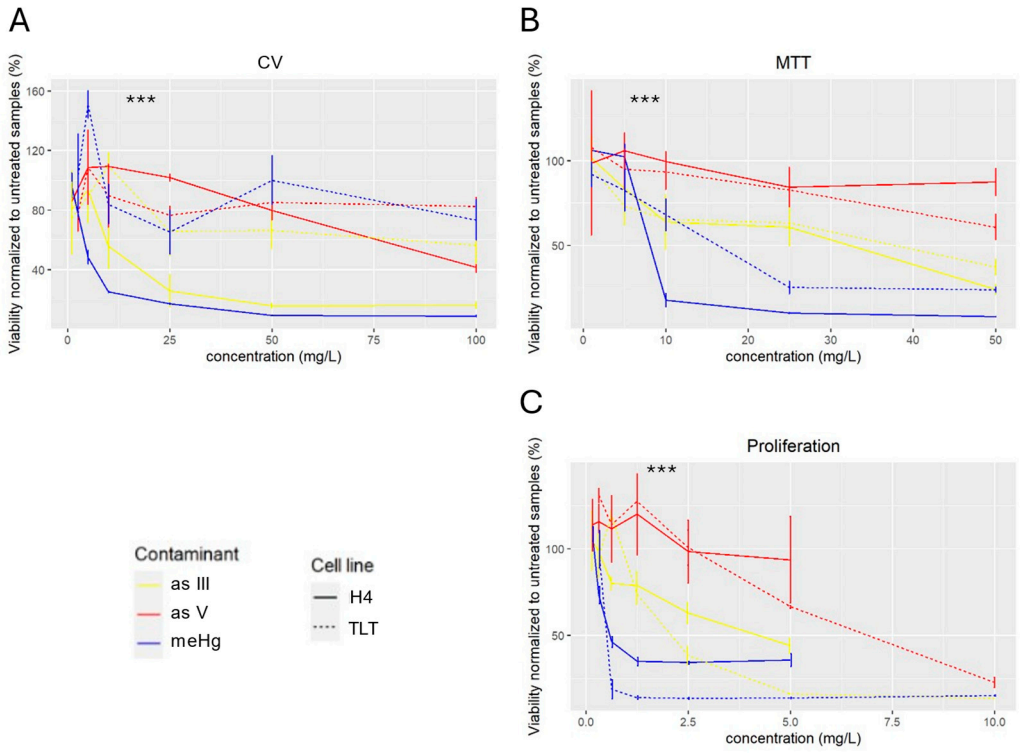

Figure 2. Assessment of the cell viability with CV, MTT and proliferation assays.

When assessing the toxicity of the heavy metals with the MTT assay we observed cytotoxicity at concentrations between 5-10 mg/L of as III and meHg in both cell lines (Figure 2B). Additionally, TLT cells showed significant viability reduction at 50 mg/L of as III (Figure 2B), while H4 cells exhibited no cytotoxicity at any tested concentration (Figure 1B).

 doi:10.20944/preprints202412.0373.v1

We observed significantly lower viability in proliferating samples treated with 2.5 mg/L of as III and meHg (Figure 2C). Similar to the MTT assay, the cytotoxic effect in proliferative samples on TLT cells was seen at higher concentrations of as V compared to as III and meHg, specifically at 10 mg/L (Figure 2C).

3.2. ROS

With the exception of two samples (1mg/L *as III* and 10 mg/L *as V*), we observed significantly higher ROS production in the rest of the H4 cells treated with the various concentrations of heavy metals. In case of the TLT cells, we observed an increase in ROS production in all the samples, with a higher magnitude to that measured in H4 cells (up to 6-fold in TLT and 3-fold in H4) (Figure 3).

We observed a concentration-response dependence in the samples treated with *as III* and *as V*, but not in those treated with *meHg*.

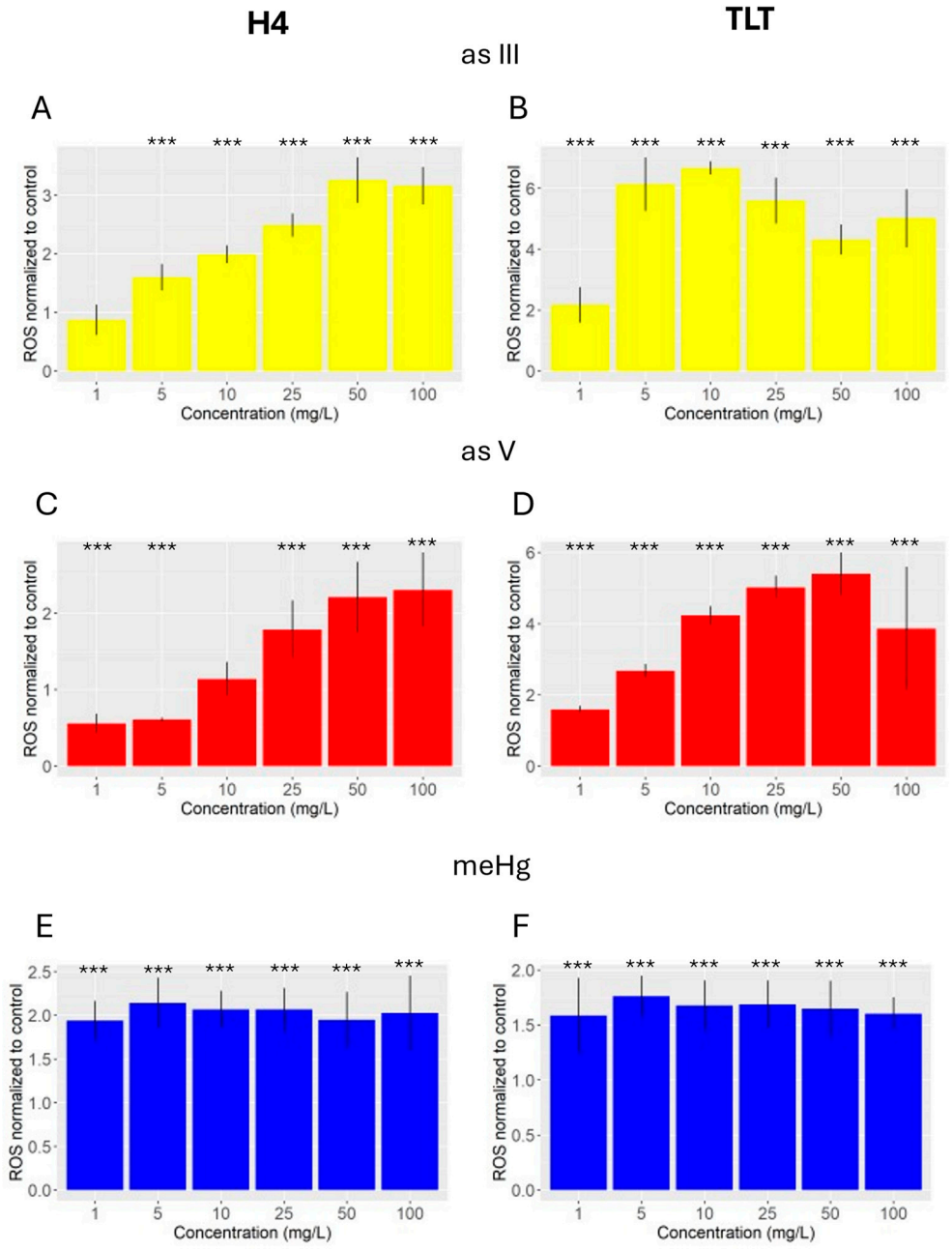

Figure 3. ROS production.

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3.3. H2ax

All the treated samples in both cell lines had higher number of exposed histones than the untreated samples with the exception of the samples treated with 5 and 10 mg/L of *meHg* (Figure 4). The cell number of these samples was lower, which is in line with the previous observations in 3.1 Cytotoxicity, where we observed a significantly higher toxicity of meHg at lower concentrations than in sample treated with arsenic.

There was no significant difference among the different samples in the activated or inactivated forms of γH2ax. Yet the proportion of the inactivated γH2ax form was higher (Figure 4).

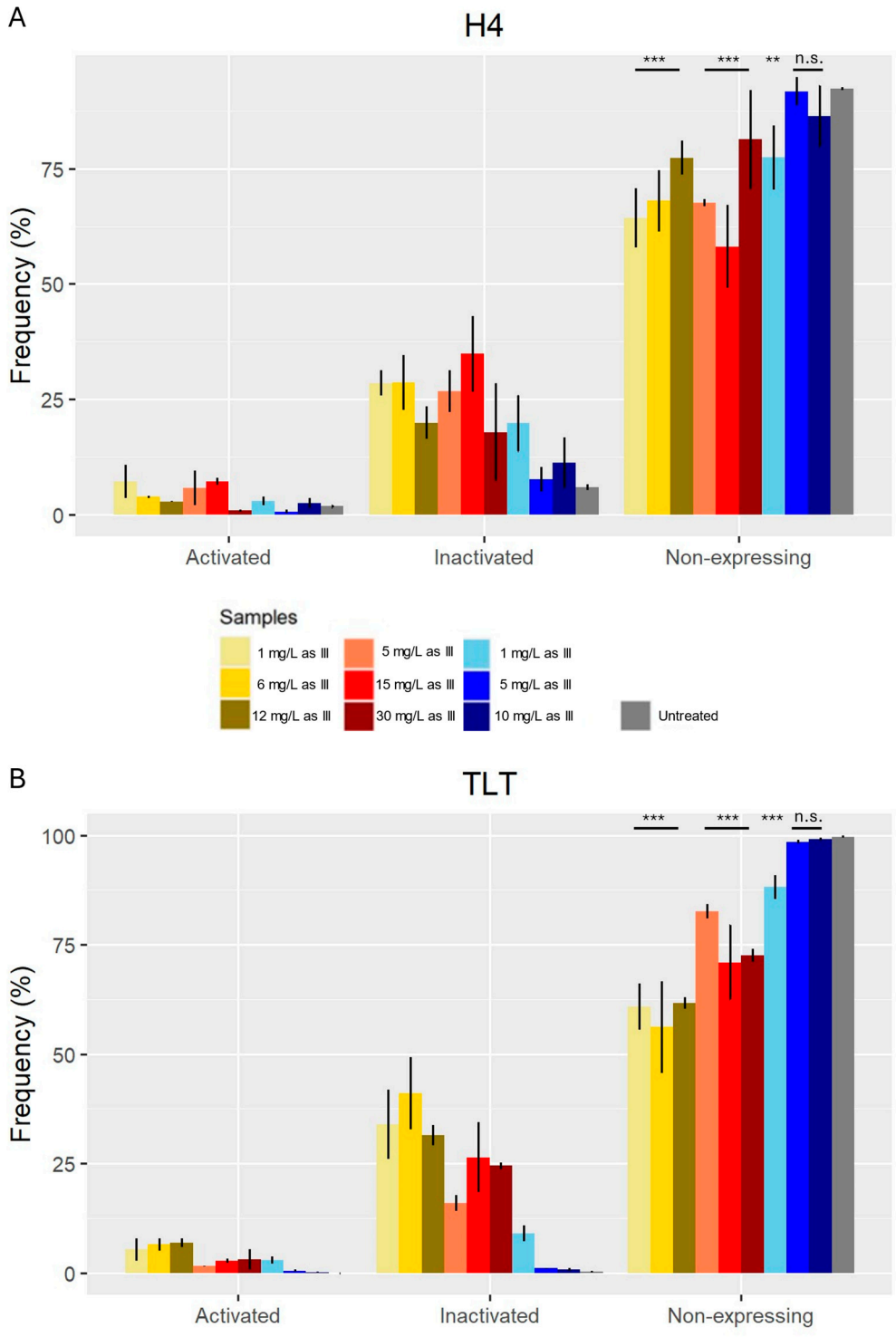

Figure 4. H2ax test on both cell lines.

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3.4. Cell Cycle

When exposing the H4 cells to 12 mg/L of *as III,* we measured a significantly higher number of cells in the G2/M phase than in the untreated samples (Figure 5A). While, H4 cells treated with 10 mg/L of *meHg* had significantly higher number of cells in the S phase and G2/M than in the untreated samples (Figure 5A).

In case of the TLT cells, we observed a significantly higher number of cells in the G2/M phase in samples treated with 5 and 15 mg/L of *as V* (Figure 5B).

We didn't observe any other significant changes in the cell cycle of the rest of the treated samples in comparison with the untreated samples.

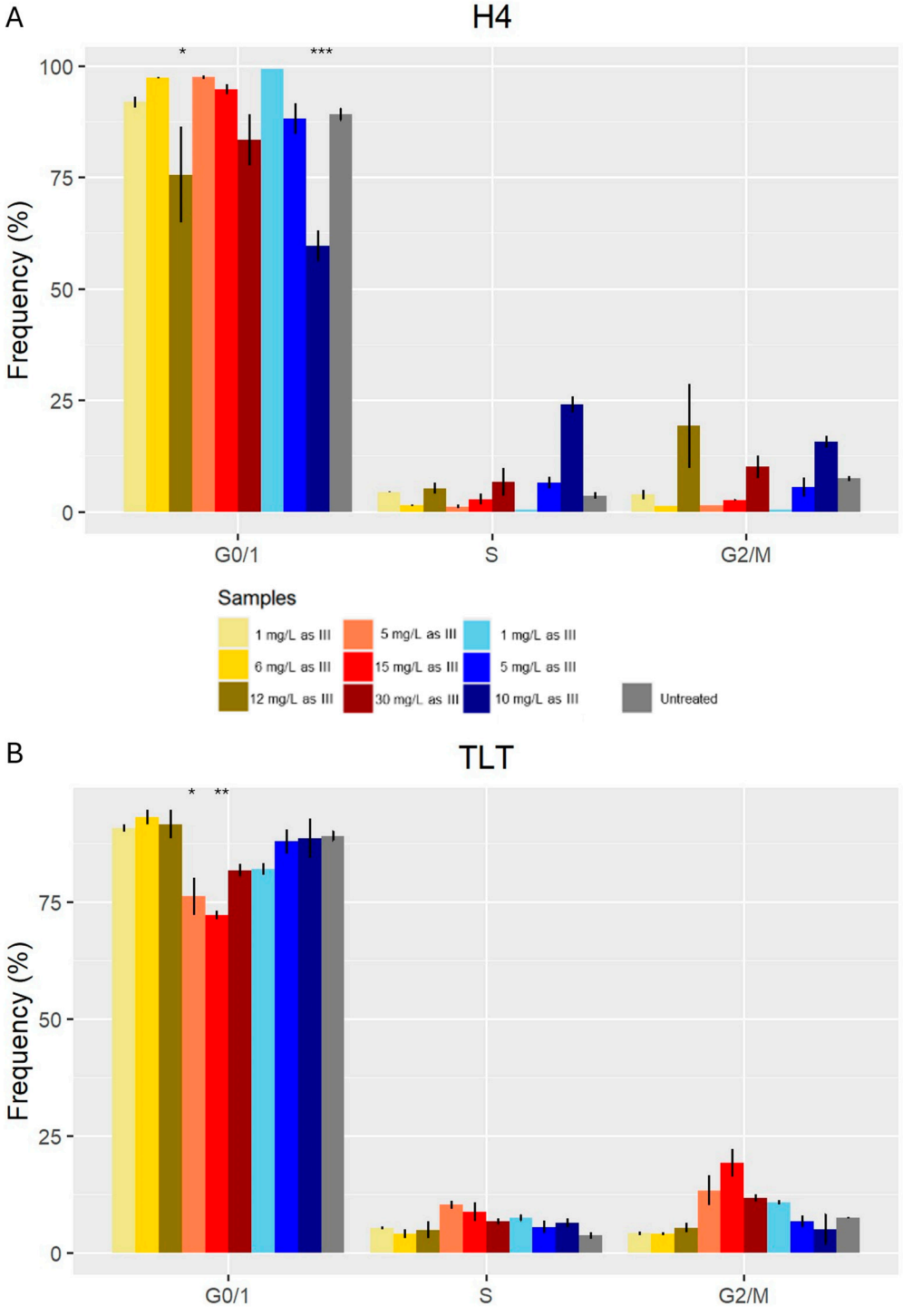

Figure 5. Cell cycle of both cell lines.

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3.4. Apoptosis

We measured a significantly higher number of H4 cells undergoing apoptosis in samples treated with 10 mg/L of *meHg* than in the untreated samples (Figure 6A). We observed a characteristic concentration-response relationship between the heavy metal concentration and the number of apoptotic cells.

The apoptotic events were significantly more frequent in TLT cells than in H4 cells (Figure 6A). Samples treated with 12 mg/L of *as III*, 15 and 30 mg/L of *as V*, and 10 mg/L of *meHg* have significantly higher number of apoptotic cells (Figure 6B). TLT cells had also concentration-response relationship between the heavy metal concentration and the number of apoptotic cells.

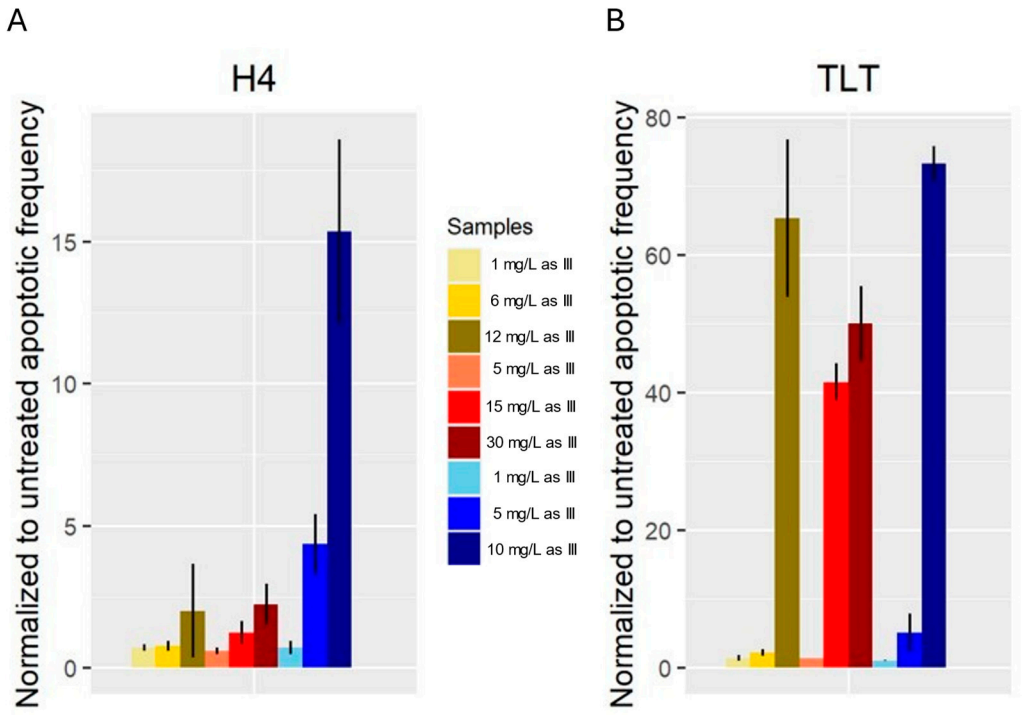

Figure 6. Apoptotic rate of the various treatments.

3.4. Cell Shape

We observed a significantly higher number of H4 cells that change their native shape after treating them with 5 mg/L of *as V* and 10 mg/L of *meHg* (Figure 7A).

The change on the cell`s native shape was more pronounced in TLT cells, more concretely, the samples treated with 6 and 12 mg/L of *as III*, 30 mg/L of *as V* and 10 mg/L of *meHg* had significantly higher number of cells with altered native shape (Figure 7B).

Also, in case of the TLT cells we observed a characteristic concentration-response relationship between the heavy metal concentration and the number of cells with altered native shape.

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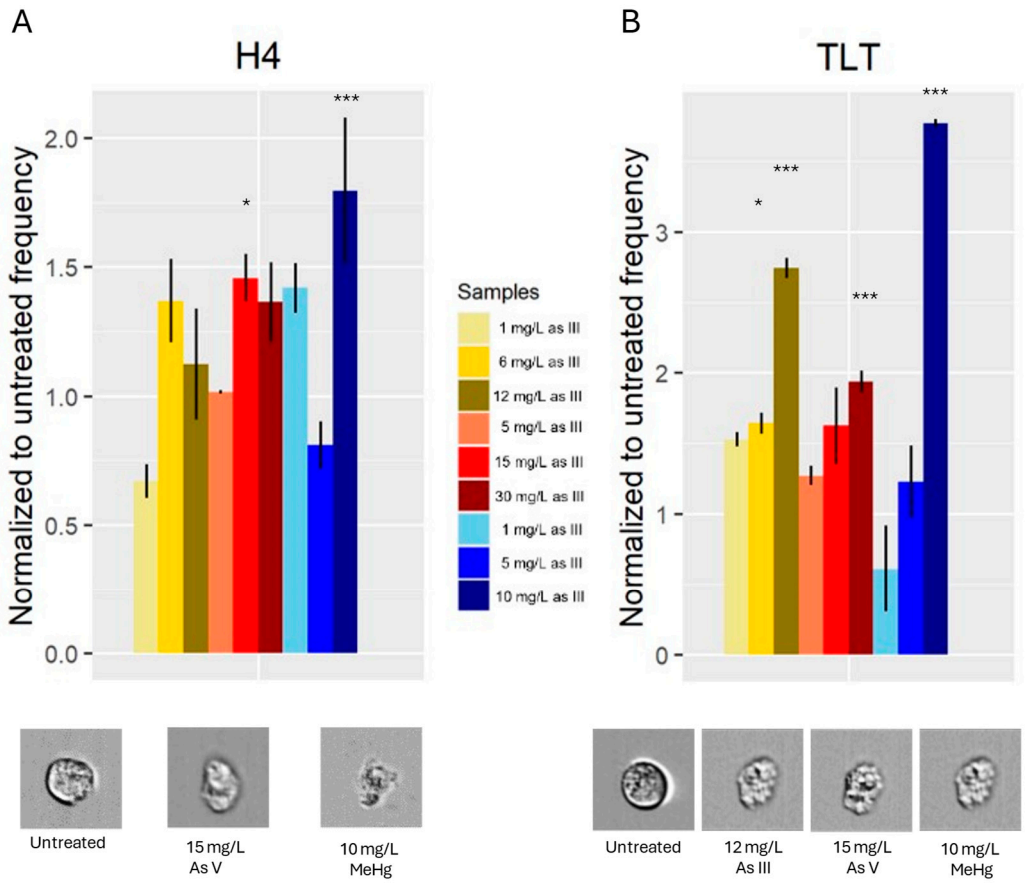

Figure 7. Cell shape change.

4. Discussion

Authors One of the most common sources for heavy metal exposure is the food, and thus, the most exposed cell types are the intestinal epithelial cells [1,2]. Once the heavy metals enter the bloodstream, blood macrophages are one of the most exposed cell types, since due to their scavenger nature uptake many of these molecules [25]. Within these terms, we selected both cell types to analyse the toxic effect of arsenic III (*as III*), arsenic V (*as V*) and methylmercury (*meHg*).

Previous studies observed that *as III*, *as V* and *meHg* were cytotoxic at different concentrations depending on the experimental setup (from 0.5 mg/L to 750 mg/L) [5,12,26–28]. We performed CV, MTT and proliferation tests to observe the cytotoxic response at similar concentrations (from 0.5 mg/L to 100 mg/L). When performing the CV, we observed cytotoxicity in both cell types when exposing them to a range from 10 to 25 mg/L of *as III* and *meHg*. Of note, we observed that the cytotoxic effect of both contaminants had a cytotoxic effect of a significantly higher magnitude in the H4 cells (Figure 2A). Also, *as III* reacts with thiol and sulfhydryl groups, which are major organic components to multiple proteins and enzymes [29]. And considering that intestinal epithelial cells have more enzymes than the blood macrophages [30,31], their impairment may trigger a more toxic response in the intestinal epithelial cells. This observation is of special interest when assessing the risk to their exposure, since intestinal epithelial cells are the most exposed to foodborne contaminants (as explained above). Among all the tested compounds, the toxic potency of *as V* was lowered as observed in previous studies [29]. We observed that the meHg potency highly differs between cell lines, but this inconsistency was only observed in this test, thus, we attribute this difference to artifacts (lower available meHg concentration because of higher cell density, a higher CV concentration at these concentrations, to name but a few possibilities) [5,12,26–28].

When analysing the toxic effect of the compounds with the MTT test we observed more comparable results between the cell types (Figure 2B). In this case, this result was expected since all

 doi:10.20944/preprints202412.0373.v1

three heavy metals impair the mitochondrial activity (via glucose-6-arsenate formation in *as V* [29], via altercation of Pyruvate dehydrogenase in *as III* [29] and overloading mitochondrial Ca^{2+} in meHg [32]), which alters the formazan reduction in the mitochondria. We observed that the viability reduction was higher in cells exposed to *meHg*, since *as III* and *as V* mechanism of action is influencing more directly the ATP production, while the *meHg* mechanism of action is more related to the mitochondrial potential (mitochondrial activity).

We observed that the toxic response in proliferative cells was at lower concentrations due to two main factors (Figure 2C). Proliferative cells need more energy to duplicate, and the heavy metals affect the ATP production [33]. Moreover, when lowering the cell number, there is a higher availability of molecules for each cell (a maximum of 4.2 × 10*13 molecules of contaminants/cell in MTT and 8.4 × 10*13 molecules of contaminants/cell in the proliferation test) [34]. We also observed that *as V* had the lowest toxic effect among the tested heavy metals which is in accordance with the literature [29,35,36].

To explore further, we investigated the ROS production in a similar concentration range as the previous tests (from 1 mg/L to 100 mg/L) (Figure 3). We observed the ROS production is increased in all the tested samples, similar to was also previously observed in different studies [37,38]. The only two samples where we didn't observe any significant increase in ROS were (1mg/L in *as III* and 10 mg/L of *as V*). Of note are also the samples treated with 1 and 5 mg/L of *as V*, that have significantly lower ROS production than the untreated sample. This may be related to the mild toxic effect of *as V*, which triggered the synthesis of superoxide dismutase (SOD) to an extent that surpass the toxic effect, lowering the ROS content within the cells [39]. Within these considerations, the previously named concentrations (1mg/L in *as III* and 10 mg/L of *as V*) may reach an equilibrium state between the production of ROS and SOD.

Based on our observations of ROS production and cytotoxicity, we established the heavy metal concentrations for subsequent tests. Since we observed that saturation occurs in samples treated with *meHg*, we anticipate *meHg* to be more toxic at lower concentrations than *as III* and *as V* in the following tests and we analysed toxicity at lower concentrations than the samples exposed to arsenic. Similarly, we expect *as III* to exhibit toxicity at lower concentrations than *as V*.

With this in mind, we determine three concentrations for each heavy metal: 1, 6 and 12 mg/L of *as III*, 5, 15 and 30 mg/L of *as V* and 1, 5 and 10 mg/L of *meHg*. We observed a significantly higher histone exposure in all the test sample, except for samples treated with 5 and 10 mg/L *meHg* (Figure 4). Considering that the samples treated with *meHg* in previous tests were the samples with the highest observed cytotoxicity, we conclude that most of the cells within these samples may undergo histone exposure and final apoptotic phase (even lysis) by the time the H2ax test was performed. In this sense, we didn't observe any significant histone exposure in comparison to the untreated samples because the resulting cells were the ones that survive the exposure. This is corroborated with the ratio of dephosphorylated (inactivated)/phosphorylated (activated) amount of H2ax. Higher amount of dephosphorylated amount of H2ax signifies the completion of the repair process in response to DNA damage [40]. Higher ratio of dephosphorylated imply that the toxicity was triggered and repair by the time the test was performed.

Since most cells were already in the late apoptosis phase (or lysis), cell cycle arrest was observed in only three samples (Figure 5). Anyhow, it is noteworthy that the cells treated with *as III* and *as V* have cell cycle arrest in the G2/M phase. While the cells treated with *meHg* have a cell cycle arrest at S and G2/M phases. Arsenic compounds trigger casitas B-lineage lymphoma (Cbl) to suppress phosphatidyl-inositol 3-kinase (PI3K/Akt) signalling, which in turn regulates p53 activation. [41–45]. Meanwhile, *meHg* enhances the expression of cell division cycle 25A (CDC25A) and P21, genes that interact with cyclin-dependent kinase (CDK) and proliferating cell nuclear antigen, leading to cell cycle arrest at the S/G2 and G2/M phases. [44,45].

In accordance of what we observed in previous tests, we observed higher rate of late apoptotic frequency in most of the treated samples in comparison to the untreated samples (Figure 6). This frequency was especially high in TLT cells, which suggest that apoptosis is the most common pathway of their programmed cell death, as observed in previous studies [46–48]. While in the H4

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samples, there were not as frequent, which suggest two possibilities: on the one hand, the H4 cell may already underwent apoptosis considering that the H4 cells were more sensitive to the compounds as it was observed in the rest of the performed tests. On the other hand, it may be possible that the H4 cells exposed to heavy metals underwent an alternative cell death pathway. It is of special interest to investigate these observations as the authors couldn't find any direct reference related to the matter.

In accordance with our previous observation, TLT cells had higher frequency on cell shape loss in comparison to the untreated cells, than in H4 cells (Figure 7). Considering that the formation of late apoptotic bodies changes the cell native shape dramatically, these results were expected. On the other hand, we didn't observe such profound change in the shape of the H4 cells, which may be, again, due to the possibilities explained above: cells altered by the compounds already lysed or due to alternative programmed cell death. Although, we couldn't compare our results to any previous work since we couldn't find any related to the topic.

Considering all the results discuss above, we now have a clearer understanding of the toxic effect of *as III*, *as V* and *meHg*. In both cell lines, *meHg* and *as III* had higher cytotoxic effect than *as V*, as observed in previous studies [5,12,26–28]. All the tested heavy metals induce to increase the ROS production, which results in DNA damage (histone exposure). Yet, the influence of these DNA damage triggered different responses in the cells, *as III* and *meHg* induced a cell cycle arrest in cell cycle in phase S and G2/M in intestinal epithelial cells, while *as V* induced a cell cycle arrest in phase G2/M in blood macrophages. The differences in the toxic effect of the heavy metals between the cell types was even higher when observing the late apoptotic frequency and native cell shape alteration. Blood macrophages have a higher apoptotic frequency and more cell native shape change than the intestinal epithelial cells. Further studies should be needed to discern the most common programmed cell death when exposing the intestinal epithelial cells to heavy metals. Thus, not only the heavy metal influences the toxic effect, but the cell type also has a significant influence in the mechanism of toxicity of the heavy metal.

Limitations

Despite the intestinal epithelial cells are the cell type with the highest exposure to heavy metals through oral intake, there is very little information regarding the toxicity of those contaminants in this cell type. We only find three references related to the toxic effect of arsenic in intestinal epithelial cells [49–51]. Further investigation should be encouraged to have a more comprehensive understanding of the mechanism of toxicity of those contaminants, which are not so rare in the food.

These are but only two cell types that are highly exposed to the foodborne contaminants. Considering the different responses observed in both cells with the same heavy metals, further experiments should aid having a more comprehensive understanding of the toxic mechanisms of the heavy metals in alternative cells. Among them, we want to point the neurons, due to the known neurotoxicity of the heavy metals [52] and hepatocytes, which are highly exposed due to their role in metabolizing xenobiotics and their high enzyme content, which are the main target of the heavy metals, as we observed before [29].

Future tests should also be performed in a shorter period after the exposure to corroborate the findings in the H2ax test. Moreover, the most common programmed cell death pathway for the intestinal epithelial cells remains unknown and should be investigated. Likewise, more concentration points should elucidate if the differences observed among cell types were due to cells already lysed or they have an alternative cell death pathway.

Considering the toxic effect, we observed, which are aligned with previous studies, special attention should be driven to understand the cancerogenic effect of these heavy metals, and their prevalence in the population.

5. Conclusions

The heavy metals induce different toxic effect considering their form and the exposed cell type. *as III*, *as V* and *meHg* induce ROS production and histone damage, but the subsequent pathways differ. TLT cells were more susceptible to suffer a cell cycle arrest at the G2/M phase and triggered

 doi:10.20944/preprints202412.0373.v1

apoptosis, while the H4 cells were more prone to a cell cycle arrest in the S phase and have an alternative cell death pathway.

Understanding the mechanisms of toxicity may help to implement effective strategies for the prevention or mitigation of the heavy metal toxicity. Thus, further investigation on the toxic effect of these compounds, especially in intestinal epithelial cells, should be performed to close this information gap.

Author Contributions: Conceptualization, E.M.; methodology, E.M.; software, E.M.; validation, E.M.; formal analysis, E.M.; investigation, E.M.; resources, E.M. and I.T.; data curation, E.M.; writing—original draft preparation, E.M.; writing—review and editing, E.M., L.K.Š., M.R., I.T., and M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 311820 (ECsafeSEAFOOD).

Conflicts of Interest: The authors declare no conflicts of interest.

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