

ORIGINAL ARTICLE

# Influence of Arsenic (III), Cadmium (II), Chromium (VI), Mercury (II), and Lead (II) Ions on Human Triple Negative Breast Cancer (HCC1806) Cell Cytotoxicity and Cell Viability

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## KEYWORDS

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Optical-Microscopy; Cell-Death;

Flow Cytometry

**ABSTRACT:** The hazardous consequences of heavy metal ions (HMIs) on human health necessitate the immediate need to probe fundamentally the interactions and cytotoxic effects of HMIs on humans. This study investigated the influence of five toxic HMIs (arsenic (As (III)), cadmium (Cd (II)), chromium (Cr (VI)), mercury (Hg (II)), and lead (Pb (II))) on human TNBC (HCC 1806) cell viability using optical microscopy, trypan blue dye-exclusion assays, and flow cytometry. The TNBC cells were exposed to varying concentrations of HMIs for 24 and 48 hours. We evaluated the influence of the concentrations and duration of HMIs exposure on TNBC cell viability. Light microscopy, cell viability assays, revealed that after 48-hour treatment of TNBC cells with  $1 \times 10^{-5}$  M of As (III), Cd (II), Hg (II), Cr (IV), and Pb (II) resulted in cell viabilities of 23%, 34%, 35%, 56%, 91% respectively, suggesting that As (III) has the greatest cytotoxicity (77% cell death) while Pb (II) showed the least (9% cell death). Furthermore, flow cytometry revealed that while Pb (II), As (III) and Cr (IV) had significant increases in cell death, Hg (II) caused a G1 arrest. Together, this study revealed that HMIs cause a differential cytotoxic effect on TNBC cells and suggest that they may have very different genotoxic targets and implications in their mutagenic potential.

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## INTRODUCTION

Humans are constantly being exposed to harmful heavy metal ions (HMIs) via occupational exposure through inhalation of contaminated air and consumption of contaminated water or polluted food items [1-4]. Elevated concentrations of HMIs in humans have been directly associated with chronic and acute health issues [2, 5]. Specifically, environmental and occupational exposure of arsenic (As) and cadmium (Cd) have been proven to be associated with human cancer, causing tumors in the lung, skin, urinary bladder, liver, sinonasal, and possible implications in renal and prostate carcinogenesis [6-9]. Exposure to elevated levels of As, mercury (Hg), and Cd, can cause many health ailments [10-12]. Lead (Pb) has been known to affect the bone marrow, liver and the reproductive systems of both males and females [13, 14]. Toxic elements and their components have negative effects on cellular organelles and components [15, 16]. Several HMIs can induce carcinogenic effects on normal healthy cells [6, 17-19]. Recent study has investigated the influence of some metal ions on TNBC cells [20]. However, little is known about the effect of several potentially toxic heavy metal ions on TNBC cells.

Breast cancer (BC) is a common disease characterized by activation of multiple signal pathways, which stimulate growth, proliferation, inhibit apoptosis, and promote the formation of new blood vessels, as well as invasion and metastasis [21, 22]. According to WHO, BC related deaths represent 16% of all types of cancer deaths globally, and one in fifty-three women will develop BC by the age of 49 yr, after which the breast cancer probability rate increases [23]. Breast cancer constitutes a group of multiple diseases characterized by different morphologies, biological behaviors, forms of presentation and clinical evolution [21], and contains various genetic alterations that can be classified into five distinct molecular subtypes based on DNA microarray expression [23-25]. A new subtype has been recently

identified as “*claudin-low*”, which appears to be enriched for stem cell markers and cells capable of forming new tumors [26, 27], and these subtypes respond differently to therapy and are associated with various outcomes, with the shortest survival rates observed in patients who have basal-like and HER-2 overexpressing subtypes [28, 29]

Triple negative breast cancer (TNBC) comprises a heterogeneous subgroup of tumors including but not limited to those classified as basal-like and *claudin-low* subtypes by expression profiling [26, 27, 30]. These tumors often behave as interval cancer [31] and are detected mostly through clinical examination rather than with a mammogram or an ultrasound [32], which indicates rapid growth and tissue density similar to normal tissue [22]. Triple negative (TN) tumors are defined according to Perou’s molecular classification as tumors that neither express hormone receptors (progesterone and estrogen) nor overexpress human epidermal growth factor receptor 2 (HER-2) [22], which accounts for approximately 15% of all breast cancer cases [22, 26, 30]. These receptors are key targets of chemotherapeutics in the treatment of breast cancer, which are void in TNBC cells. Triple negative breast cancer is an aggressive phenotype that shows substantial overlap with basal-type and BRCA1-related breast cancers [33] and is the only cancer to have tumors that are estrogen receptor (ER) negative, progesterone receptor (PR) negative, and HER-2 negative [28, 29], causing treatment by directing agents or hormone therapy not amenable due to this property [15, 30, 32, 34]

Due to this lack of molecular target, systematic treatment options for patients with TNBC are limited to cytotoxic chemotherapy [35] as well as other cytotoxic regimens, which are also active, indicating that TNBC is chemosensitive [33]. Previous studies have highlighted several potential therapeutic effects of cytotoxic or

metal compounds in the treatment of cancer [6, 36, 37], which have demonstrated promising activity [38, 39].

The overarching goal of this study was therefore to investigate the cytotoxic effects of selected toxic HMIs (As (III), Cd (II), Cr (VI), Hg (II), and Pb (II)) on TNBC cells. There is evidence that the selected HMIs for this study affect multiple cellular processes including cell proliferation, differentiation, and apoptosis [40]. They also have the ability to induce both replication-dependent DNA double strand breaks and homologous recombination, as well as induce apoptosis in other solid cancer lines, including breast cancer cells [21, 28, and 33]. These HMIs were further selected for the study because of their high risk tendency to cause neurotoxic effects, induce carcinogenesis and cause human tumors [6, 17-19].

## MATERIALS AND METHODS

### *Chemicals and Cell Culture*

Growth medium RPMI 1640 containing 2.05 mmol/L L-glutamine, trypsin 0.25% solution, and phosphate buffered saline (PBS) were purchased from GE Healthcare Life Sciences (Logan, Utah). Mini-PROTEAN TGX Gels (4-15%) were purchased from Bio-Rad Laboratories. PARP  $\frac{1}{2}$  (H-250), rabbit polyclonal IgG antibody p53 (DO-1) and mouse monoclonal IgG were purchased from Santa Cruz Company. Anti-rabbit IgG peroxidase and anti-mouse IgG peroxidase were purchased from Sigma Aldrich. The human breast cancer cell lines (HCC 1806) were purchased from American Type Culture Collection (Manassas, VA). This cell line was derived from a 60-year-old African-American woman on July 31, 1995, who suffered from a primary squamous cell carcinoma. The tumor was categorized as a grade 2 tumor.

### *Cell Treatment and Protocol*

Stock solutions ( $1.0 \times 10^{-3}$  M) of each HMIs were made from its corresponding 1000 ppm standard stock solution and dissolved in growth media to produce treatment solutions of the same concentration. HMI-solvent control treatment solutions were made by dissolving specific volumes of 2% HNO<sub>3</sub>, corresponding to volumes of acid used to make treatment solutions of HMIs. The HCC1806 cells were split and placed in the incubator for 24 hours. Pre-treatment images of the cells were taken with a microscope (Olympus 1x71) at a magnification of 10X. Old media and non-adhesive cells (dead cells/debris) were removed by aspiration. The remaining adherent cells were rinsed with 1 mL aliquots of 1X phosphate buffered saline (PBS) and fresh cell medium was added to each corresponding cell plate. A known concentration of the HMIs was titrated in 100  $\mu$ L aliquots into each of the cell plates and buffered with 900  $\mu$ L of fresh medium to obtain a total volume of 1mL, and final treatment concentrations of  $1 \times 10^{-5}$  M. The media control plate contained 1000  $\mu$ L of media, received no HMI treatment, and are used to take into account the effects of the solvent acid on the cells as compared to the HMIs dissolved in the solvent acid. Treated TNBC cells were placed in a humidified incubator for 24 and 48-hour time-points at 37°C/5% CO<sub>2</sub>.

### *Cell Viability Assay*

After 24 and 48-hour incubation period, the cell viability was assessed by the standard trypan blue exclusion assay protocol using a TC10 automated cell counter (BioRad). In brief, 20 $\mu$ L of the trypan blue dye was added to 20 $\mu$ L of treated TNBC cells. Aliquots of 10 $\mu$ L of cell/dye suspension were added to each side (A and B) of cell counting slide (BioRad). Both viable (transparent) and nonviable (blue) cells were counted.

Each side of the cell-counting slide was read in the TC10cell counter in triplicates and data was recorded.

### ***Cell Cycle Analysis and Statistical Data Analysis of Cell Viability***

After a 24-hour incubation period with HMI or control solvents, the treated cells were pelleted by centrifugation at 900g for five minutes. Cell pellets were loosened and washed twice in 1XPBS, then centrifuged at 900g for five minutes. Re-suspended cells were then fixed in 2 mL of cold 70% ethanol, and stored temporarily at 4°C. For staining, cell pellets were centrifuged and re-suspended in 1mL of a propidium iodide/RNase (50µg/ml:100µg/mL) staining solution. A total of 30,000 cells were acquired using BD Accuri C6 Cytometer (BD Biosciences) and the cell cycle profiles analyzed using the FCS Express 5 Plus software (DeNovo Software).

Data were presented as means ± SDs. Comparisons between media control, metal solvent controls and HMI treated cells were assessed to analyze the differences by the student's paired *t*-test ( $P < 0.05$ ) was considered statistically significant.

## **RESULTS AND DISCUSSION**

### ***Cytotoxic Response of TNBC Cells to Heavy Metal Ions (HMIs) Treatments***

The initial study investigated the influence of concentration and duration of HMI exposure. TNBC were exposed to three different concentrations ( $1 \times 10^{-7}$  M,  $1 \times 10^{-6}$  M, and  $1 \times 10^{-5}$  M) of As (III), Hg (II), Cd (II), Cr (VI) and Pb (II) for a 24, 48, 72 and 144-hour time course. At the end of each time the cytotoxicity was evaluated by light microscopy. The result of the time course study revealed significant cytotoxic effects on TNBC cells within the first 24 hours of exposure for all investigated HMIs (data not shown). This study also revealed that  $1 \times 10^{-5}$  M is the

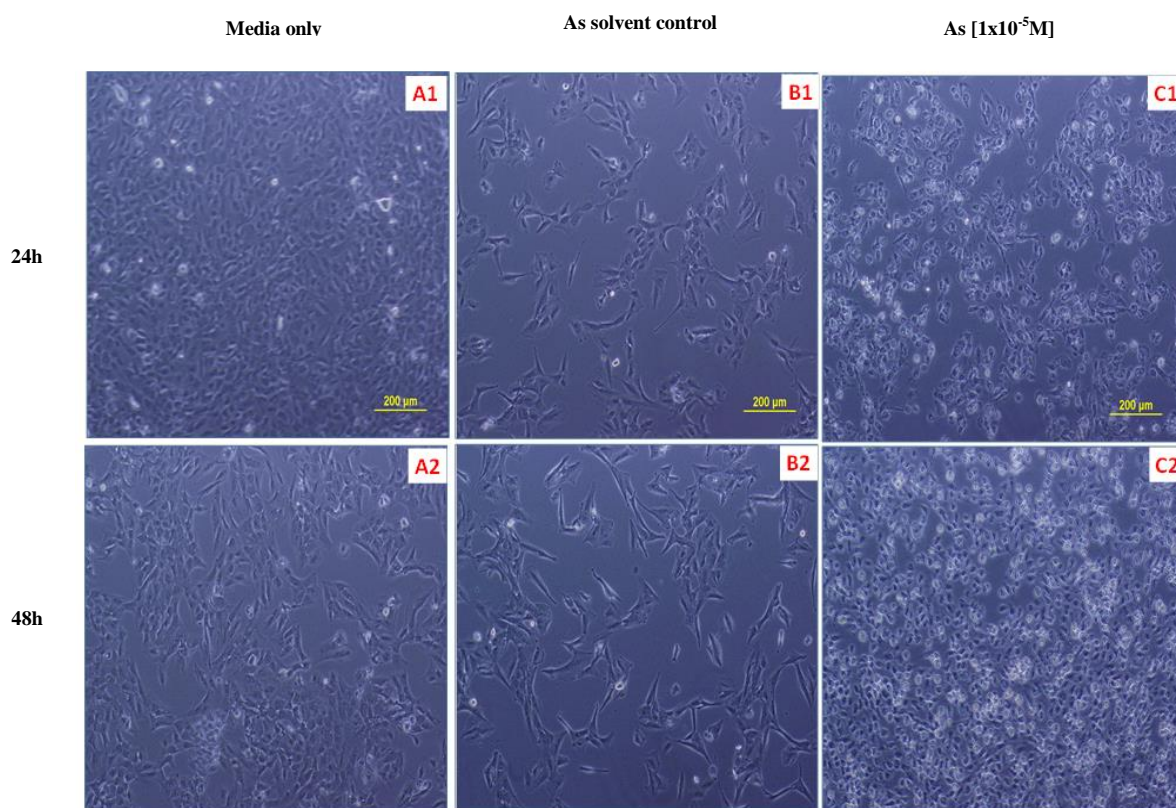
concentration with greatest influence on TNBC cell viability and therefore all further studies of each HMI were conducted using  $1 \times 10^{-5}$  M concentration.

The influence of HMIs on TNBC cell viability was evaluated for each metal ion at  $1 \times 10^{-5}$  M concentration. Human TNBC cells were exposed to each metal ion separately for 24 and 48 hours and the cytotoxic effects compared to the no treatment (media only) and metal solvent (2% HNO<sub>3</sub>) controls. Results of the cytotoxic physical cell responses revealed qualitatively that HMIs cause a differential response in TNBC cells. For instance, As (III)-treated cells displayed white, rounded floating dead cells and cell debris that increased with time (Figure 1). On the contrary, no physical change was observed from media treated cells. However, very little morphology changes were observed in the metal solvent control cells. In comparison to the physical cellular responses of As (III)-control cells, increases in physical cytotoxicity of floating dead cells was observed in the As (III)-treated cells, denoting that changes in appearance of cells were due to the effect of As (III) on TNBC cells. In addition, morphology of cells changed from uniformed and flattened appearance for non-treated cells to shriveled appearance for As (III)-treated cells indicating cell death within 24 hours of exposure. However, even deader cell accumulations of shriveled and floating cells were observed within 48 hours of As (III) exposure and continued to increase with time (data not shown).

The results of a similar study conducted for Hg (II), Cd (II), Cr (VI), and Pb (II) treated TNBC cells are shown in Figure 2 through Figure 5, respectively. The treatment of TNBC cells with Hg (II) resulted in changes in morphology and the shape of cells in the 24 and 48-hour exposure times. Hg (II) treatment of TNBC cells produced a physical response similar to that of Cr (VI) through cell elongation, followed by morphology of the cell structure into circular structures (Figure 2). Physical morphology changes were observed for Cd

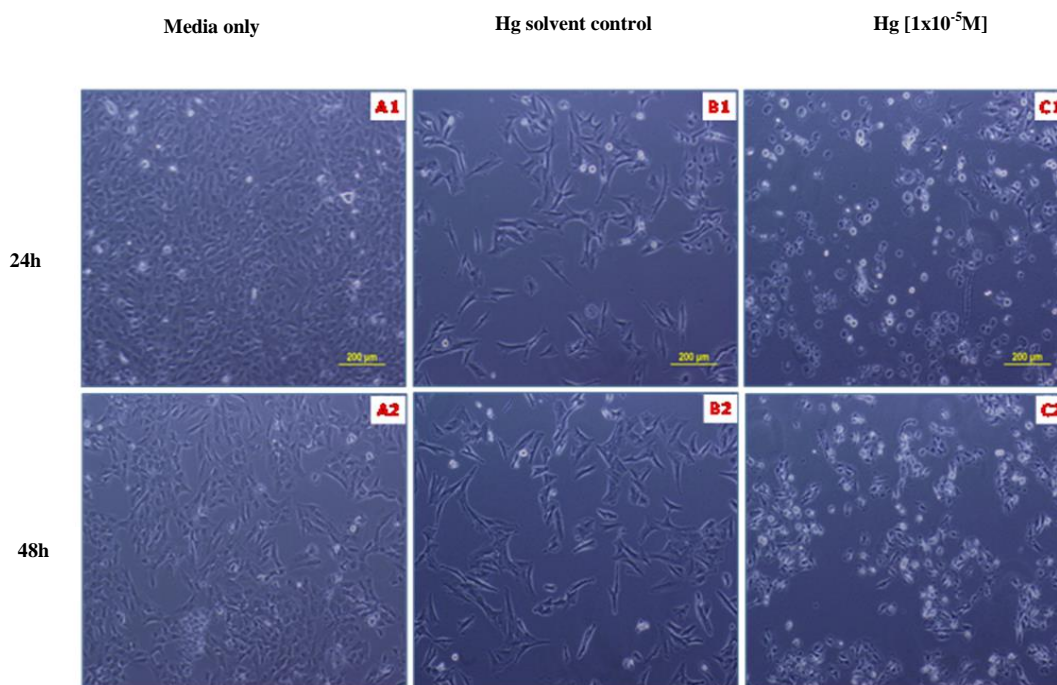
(II)-treated cells (Figure 3) demonstrating a very similar response in 24-hour exposure with that of As (III)-treated cells. Interestingly, a different physical response was observed in 48 hours of Cd (II) exposure in comparison to the response experienced by As (III)-treated cells. In the presence of Cr (VI), a slight variation was observed in comparison of media and solvent control images, as well as in comparison with the time of exposure (Figure 4). In the initial 24 hours, cell structures become elongated and deformed from

original shape. However, after another 24 hours of exposure, cell shape changes to white and rounded structures. Pb (II) demonstrated the least response in comparison to all other HMIs investigated. Images displayed slight physical response within the first 24 hours of cellular exposure (Figure 5). Cell structures became elongated and maintained this shape at the 48-hour exposure time. Interestingly, cell death was not observed in the treatment of TNBC cells with Pb (II).

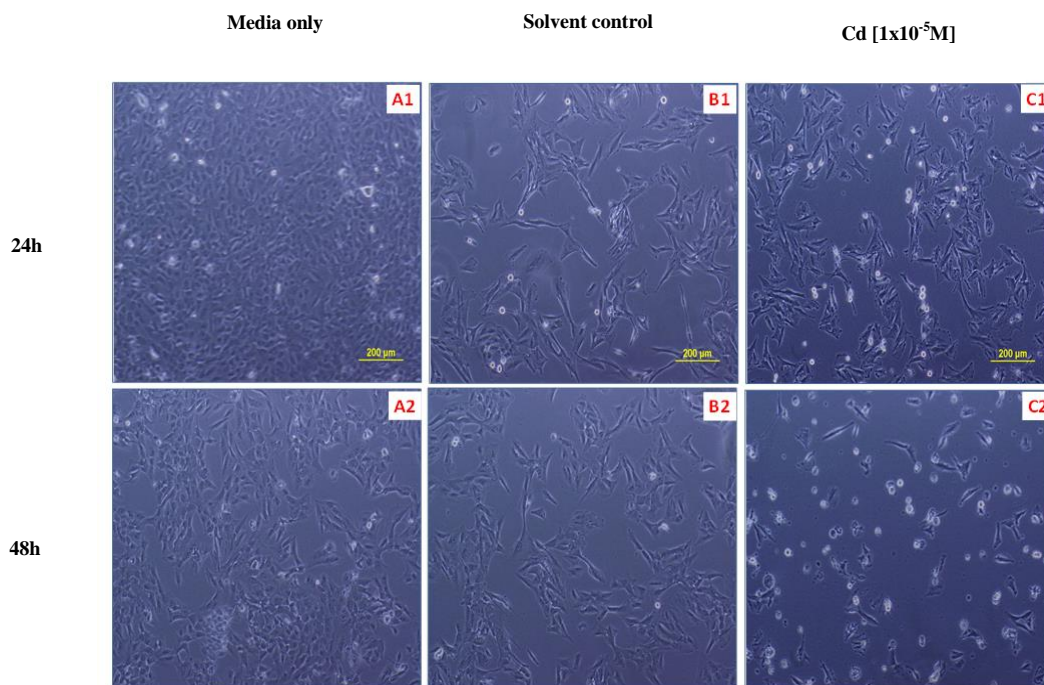


**Figure 1.** As (II) Treatment of TNBC cells at: **A1**) 24-hour media control, **A2**) 48-hour media control, **B1**) 24 hour As (III)-control, **B2**) 48 hour As (III)-control, **C1**) 24 hour As (III) treatment, **C2**) 48 hour As (III) treatment.

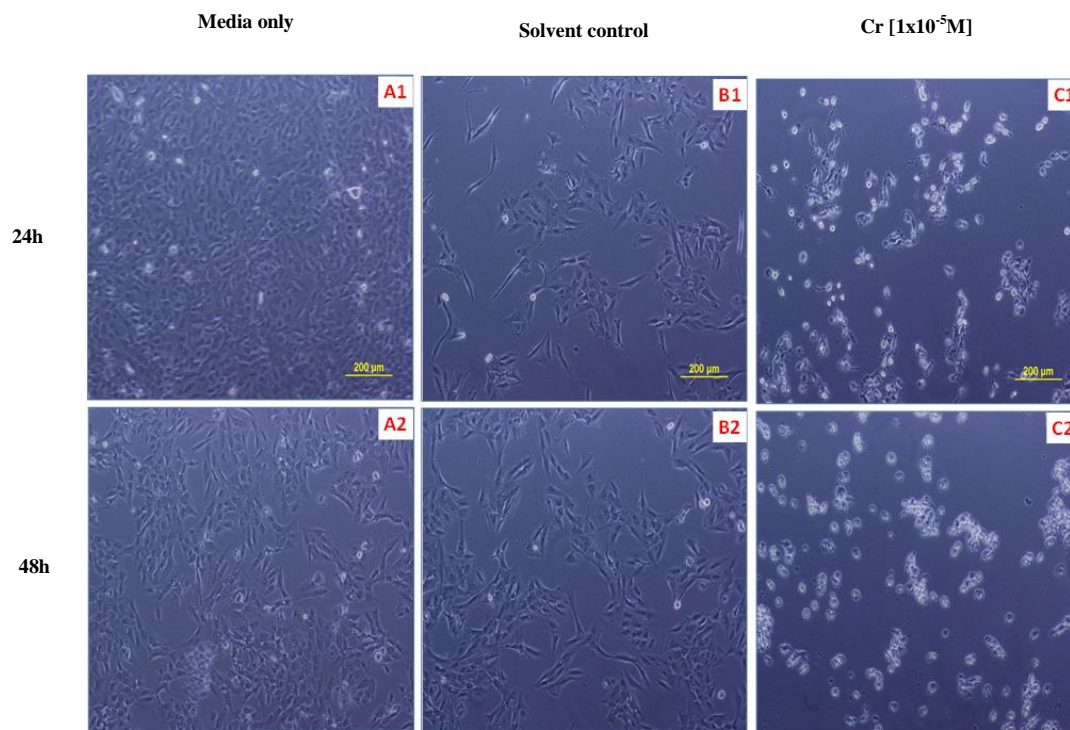




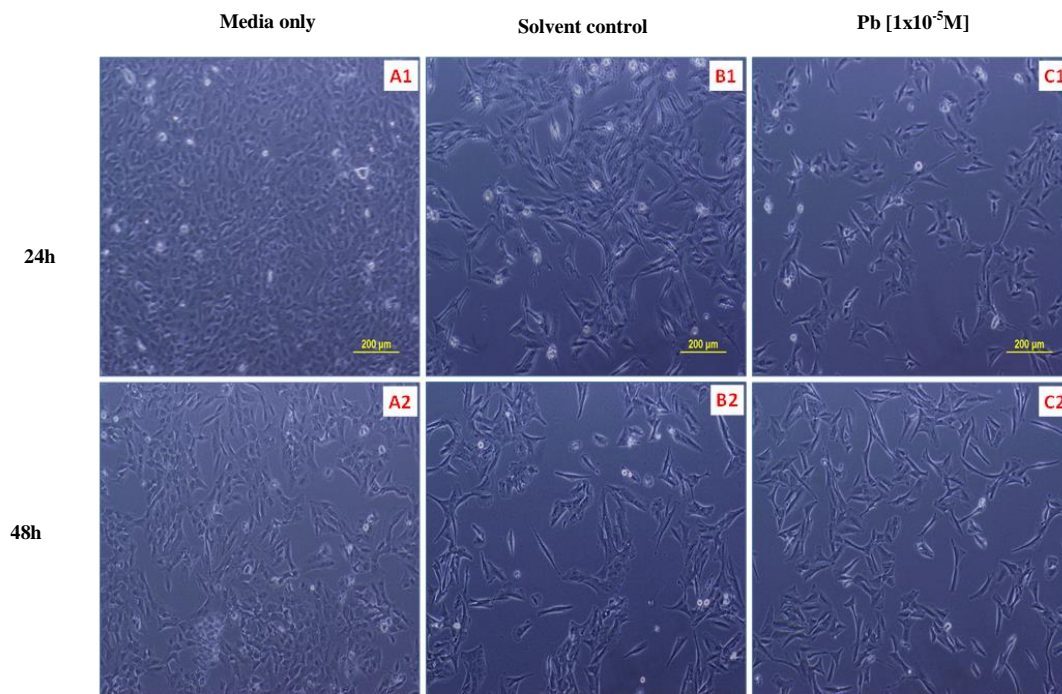
**Figure 2.** Hg (II) Treatment of TNBC cells at: **A1**) 24-hour media control, **A2**) 48-hour media control, **B1**) 24-hour Hg (II)-control, **B2**) 48-hour Hg (II)-control, **C1**) 24-hour Hg (II) treatment, **C2**) 48-hour Hg (II) treatment.



**Figure 3.** Cd (II) Treatment of TNBC cells at: **A1**) 24-hour media control, **A2**) 48-hour media control, **B1**) 24-hour solvent-control, **B2**) 48-hour solvent-control, **C1**) 24-hour Cd (II) treatment, **C2**) 48-hour Cd (II) treatment.



**Figure 4.** Cr (VI) Treatment of TNBC cells at: **A1** 24-hour media control, **A2** 48-hour media control, **B1** 24-hour solvent-control, **B2** 48-hour solvent-control, **C1** 24-hour Cr (VI) treatment, **C2** 48-hour Cr (VI) treatment.



**Figure 5.** Pb (II) Treatment of TNBC cells at: **A1** 24-hour media control, **A2** 48-hour media control, **B1** 24 hour solvent-control, **B2** 48 hour solvent-control, **C1** 24 hour Pb (II) treatment, **C2** 48 hour Pb (II) treatment.

### **Results of Cell Viability Assays of Heavy Metal Ion treatments in TNBC Cells**

Cell viability was performed, using  $1 \times 10^{-5}$  M of each HMI and metal solvent controls. TNBC cells were cultured in the presence of these HMIs for 24 and 48-hour periods. Cell viability was determined based on the trypan blue exclusion assay. Each point represents a mean value of six readings using the TC10BioRad cell counter. The viability of media control cells was designated as 100%, and the treatment cells were expressed as percent compared to the controls. A slight decrease in cell viability of TNBC cells treated with As (III) was observed with a minimal cell death percentage of 9% during the initial 24 hours of treatment (Figure 6A). However, a significant ( $P < 0.05$ ) 77% cell death was observed during the 48-hour exposure (Figure 6B), confirming the time-dependent nature of the cytotoxic effects of HMIs. These data suggest that As (III) is highly cytotoxic to human TNBC (HCC1806) cells. With the exception of Pb (II), significant ( $P < 0.05$ ) TNBC cell deaths were obtained at 48 hours of exposure. Among all five HMI treatments, As (III) showed the most pronounced cytotoxic activity against TNBC cells, being two-fold more potent than Cr (VI) treatments after 48 hour exposures. Cell viability due to Hg (II) showed approximately 10% decrease but significant ( $P < 0.05$ ) in live cell percentages of a 24-hour treatment (Figure 6C). This result is consistent with the results of the 24-hour treatment of As (III), Cd (II) and Cr (VI) on cell viability of human TNBC cells. In a manner closely resembling As (III) ions, a significant increase in cytotoxicity and cell death was observed after 48 hours of treatment of Hg (II) ions on TNBC cell viability. This is evident as Hg (II) resulted in cell death greater than 60% (Figure 6D), indicating a substantial cytotoxicity only surpassed by As (III) treatment.

Cell viability was analyzed after 24 hours of treatment and displayed little to no decrease in the percentage of live cells (Figure 6E). There were significant cytotoxic effects observed in the light microscopy images, however, there is relatively no response in the cell viability of TNBC cells with Cd (II). There is an indication of possible cellular arrest, as opposed to cell death. After 48 hours of Cd (II) exposure (Figure 6F), a 65% reduction in live cell percentage was observed with approximately 15% more cell death than the standard value of 50% cell death or greater. The first 24 hours of Cr (VI) treatment on TNBC cells (Figure 6G) resulted in less than 10% cell death, displaying no variation in the results calculated from the cell viability of the media control. The notable increase in cellular death was indicated by a reduction in live cell percentage of over 40% after 48 hours of treatment (Figure 6H). In comparison to the cytotoxic effects of As (III) and Cd (II) on TNBC cells, Cr (VI) displayed a moderate toxicity on the physical response of TNBC cells after 48 hours of treatment. In the case of cell viability, Pb (II) ions demonstrated no decrease in live cell percentage in 24 hours of treatment of TNBC cells and displayed insignificant variation in comparison to solvent control cells (Figure 6I). In addition, similar results were observed even after 48 hours of treatment of Pb (II) on cell viability, indicating that Pb (II) display the least cytotoxic effect on TNBC cells (Figure 6J).

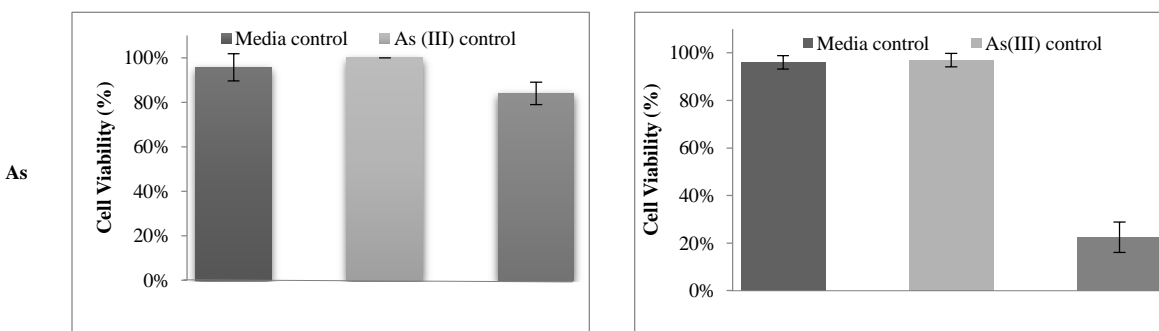
### **Results of Cell Cycle Analysis of Heavy Metal Ion treatments in TNBC Cells**

Cell cycle analysis was performed, using  $1 \times 10^{-5}$  M of each HMI and metal solvent controls and subjecting the cells to flow cytometry. TNBC cells were cultured in the presence of these HMIs for a 24-hour period and then



collected, fixed, and stained with propidium iodide to measure and quantify DNA content in order to establish the percentages of cells in the sub-G1 (i.e., apoptotic cells or cellular debris from necrotic cells), G1, S, and G2/M phases of the cell cycle. The media control cells showed essentially no change in the percentages of cells in the defined cell cycle phases after 24-hours as expected (Figure 7A). In the HMI exposed cells, the cell cycle phases were compared to the metal solvent control and revealed that the changes in the cell cycle phases were dependent on the type of HMI. The As (III) exposed cells responded with a significant increase of cells in the sub-G1 phase from 5.49% to 12.79% (Figure 7B). Interestingly, the Hg (II) exposed cells resulted in an accumulation of cells in the G1 phase from 54.19% to 68.08% suggesting a cell cycle arrest response (Figure 7C). The Cd (II) exposed cells did not show a significant change in any of the cell cycle phases as compared to the solvent control (Figure 7D), however both the solvent and Cd (II) exposed cells revealed a significant cell cycle profile change in the accumulation of cells in the G2/M phase when compared to the 24-hour media control cells (Figure 7D & Figure 7A). The Cr (VI) exposed cells resulted in a significant increase

in the sub-G1 phase from 4.82% to 13.86%, and a decrease of cells from the G2/M phase with a slight increase in cells in the G1 and S phases (Figure 7E). Interestingly, Cr (VI) solvent control caused a significant accumulation of cells into the G2/M phase when compared to the 24-hour media control cells. The Pb (II) exposed cells did not show a significant change in the cell cycle phases as compared to the solvent control (Figure 7F). These results revealed that the cell cycle response was dependent on the type of HMI, with Pb (II) showing the least changes when compared with both the solvent control and the media control cells. The Hg (II) exposed cells were the only ones to show a significant accumulation of cells in the G1 phase. The As (III) exposed cells showed a significant amount of sub-G1 cells without the other cell cycle changes caused by the solvent control when compared to the media control cells, as compared to both the Cd (II) and Cr (VI) solvent control effects on the cell cycle phases when compared to the media control cells. The dramatic cell profile changes as revealed in the histograms of both the Cd (II) and Cr (VI) exposed cells may have an effect on accurately quantifying the percentages of cells in the cell cycle phases (Figure 7D and Figure 7E



**Figure 6.** Cell Viability of TNBC after exposure to: As (III), Hg (II), Cd (II), Cd (II), Cr (VI) and Pb (II) at 24 hrs and at 48 hrs.

Data is presented as means  $\pm$  SDs. Comparisons between media control, metal solvent controls and HMI treated cells were assessed to analyze the differences by the student's paired *t*-test.  $P < 0.05$  was considered statistically significant.

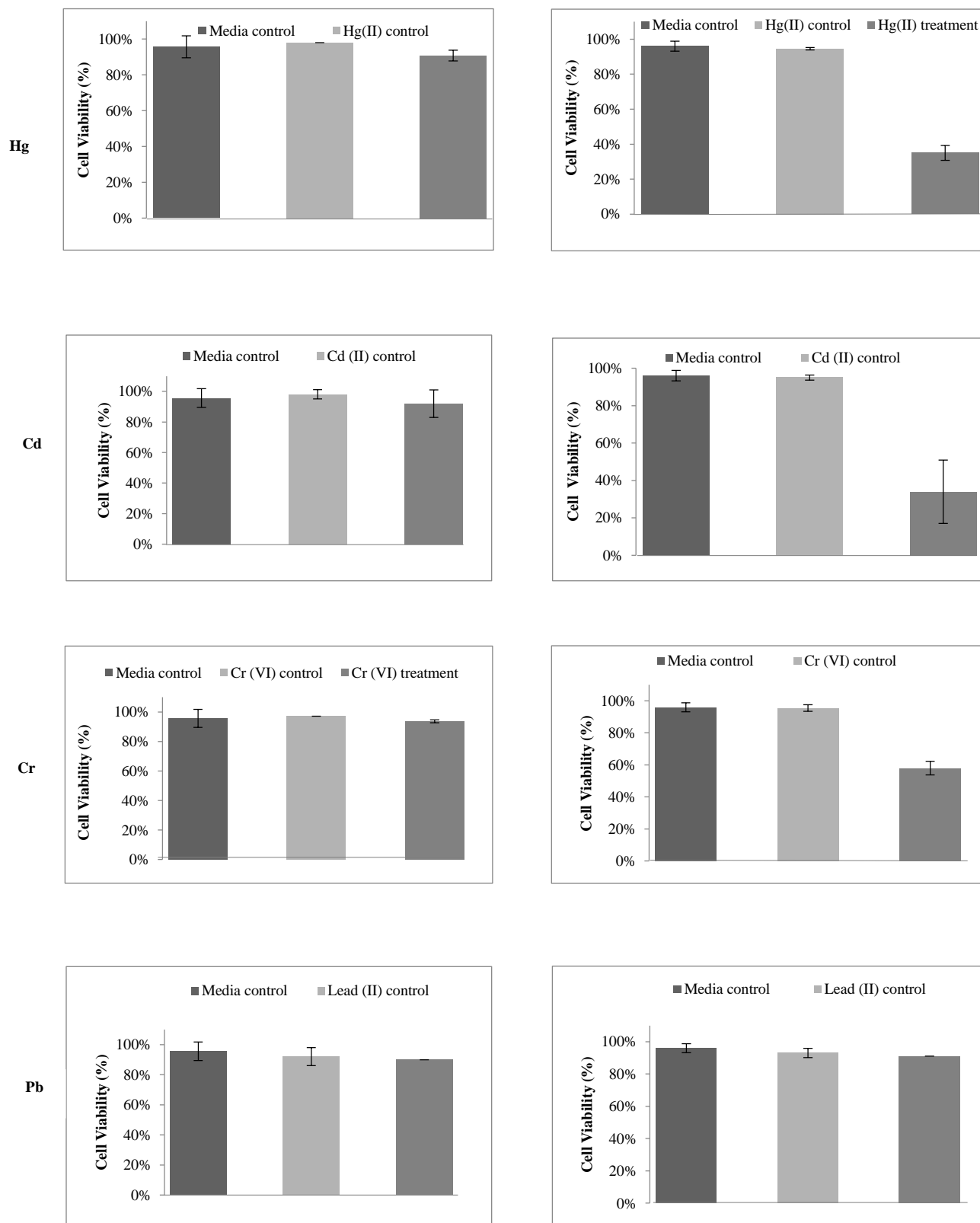
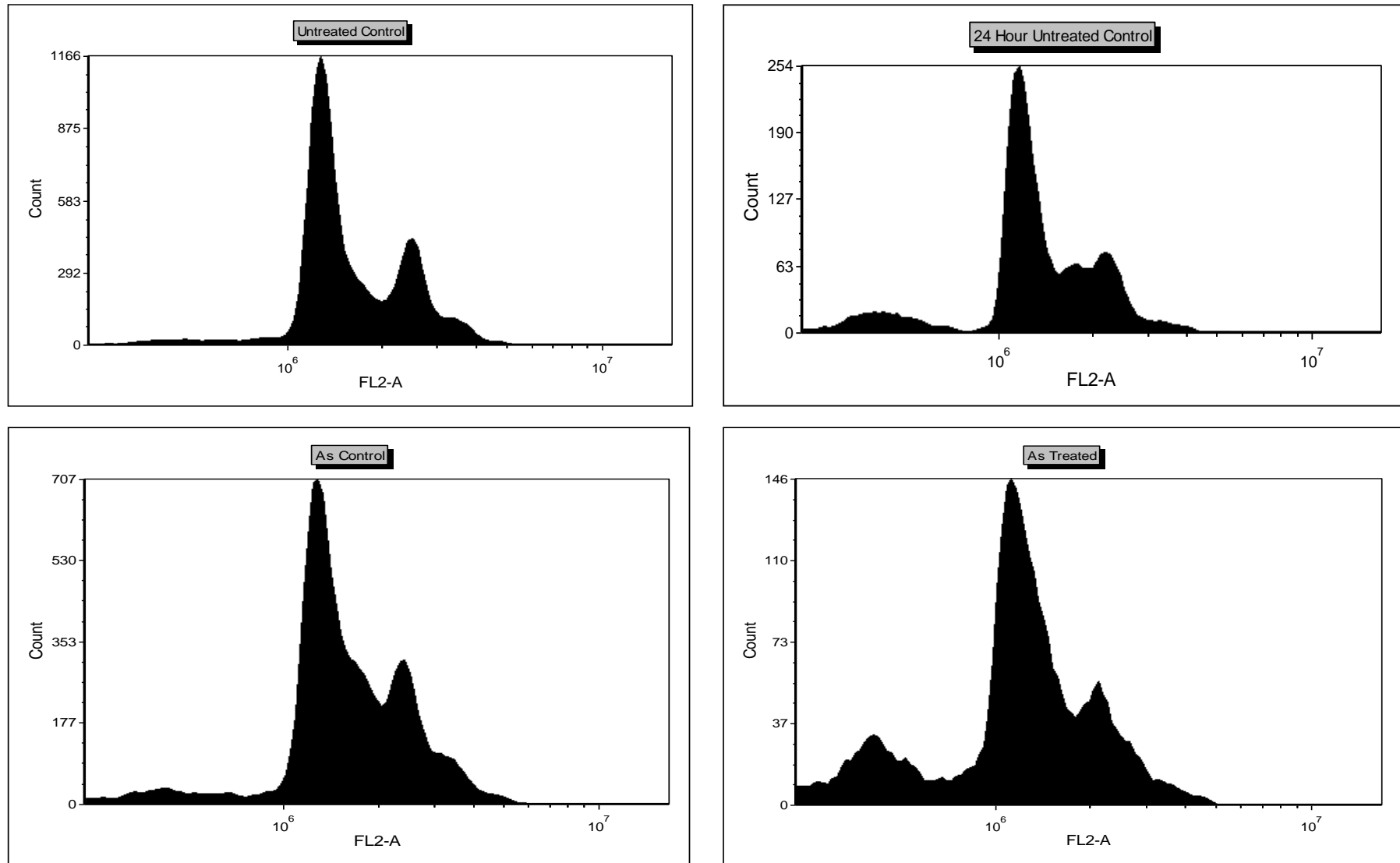


Figure 6. Continued



**Figure 7:** Result of Cell Cycle Analysis of Heavy Metal Ion treatments in TNBC Cells at 24 hrs. for: **A)**Control, **B)** As (III), **C)**Hg (II), **D)** Cd (II), **E)** Cr (VI), and **F)** Pb (II) ions.

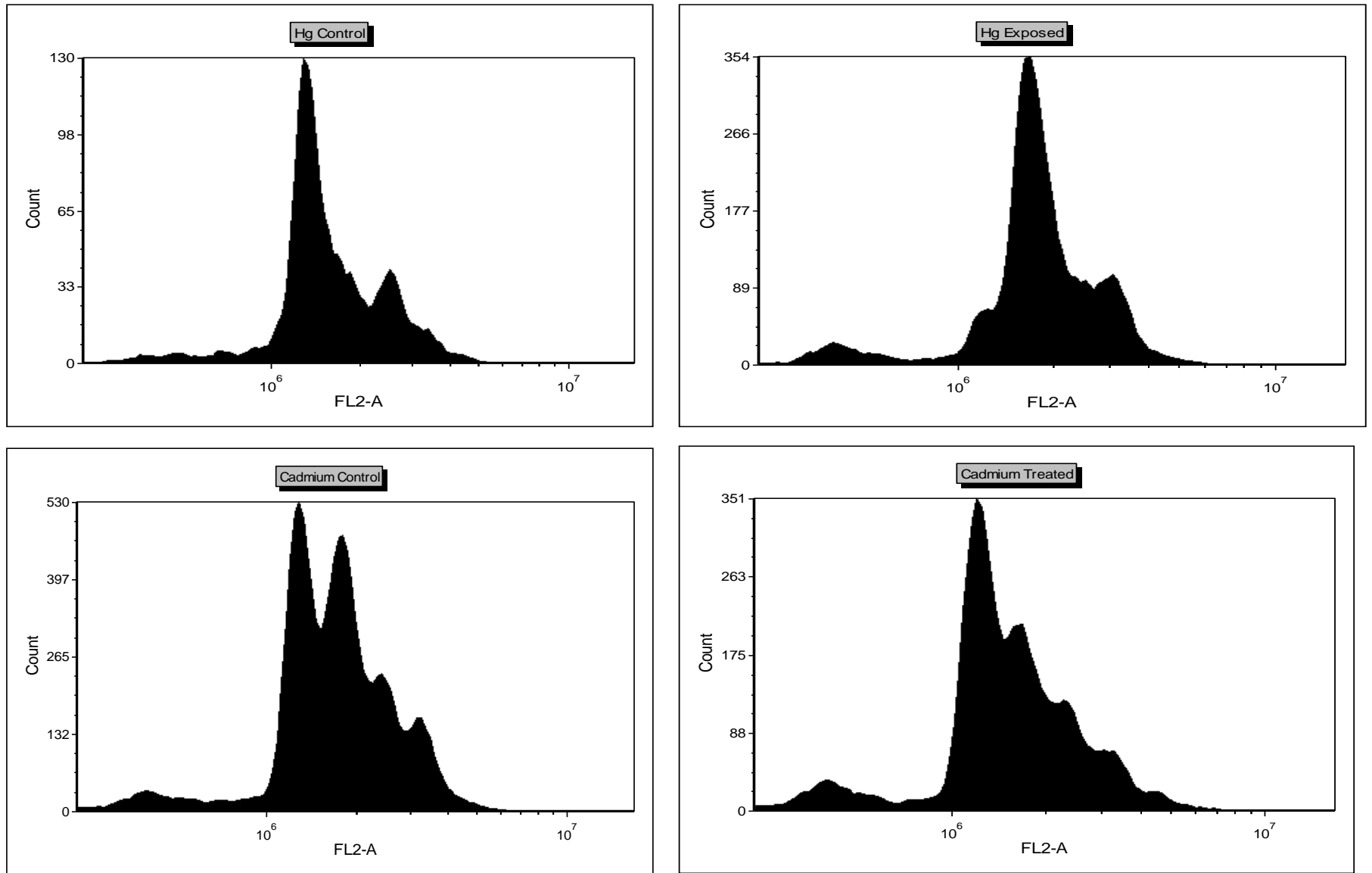


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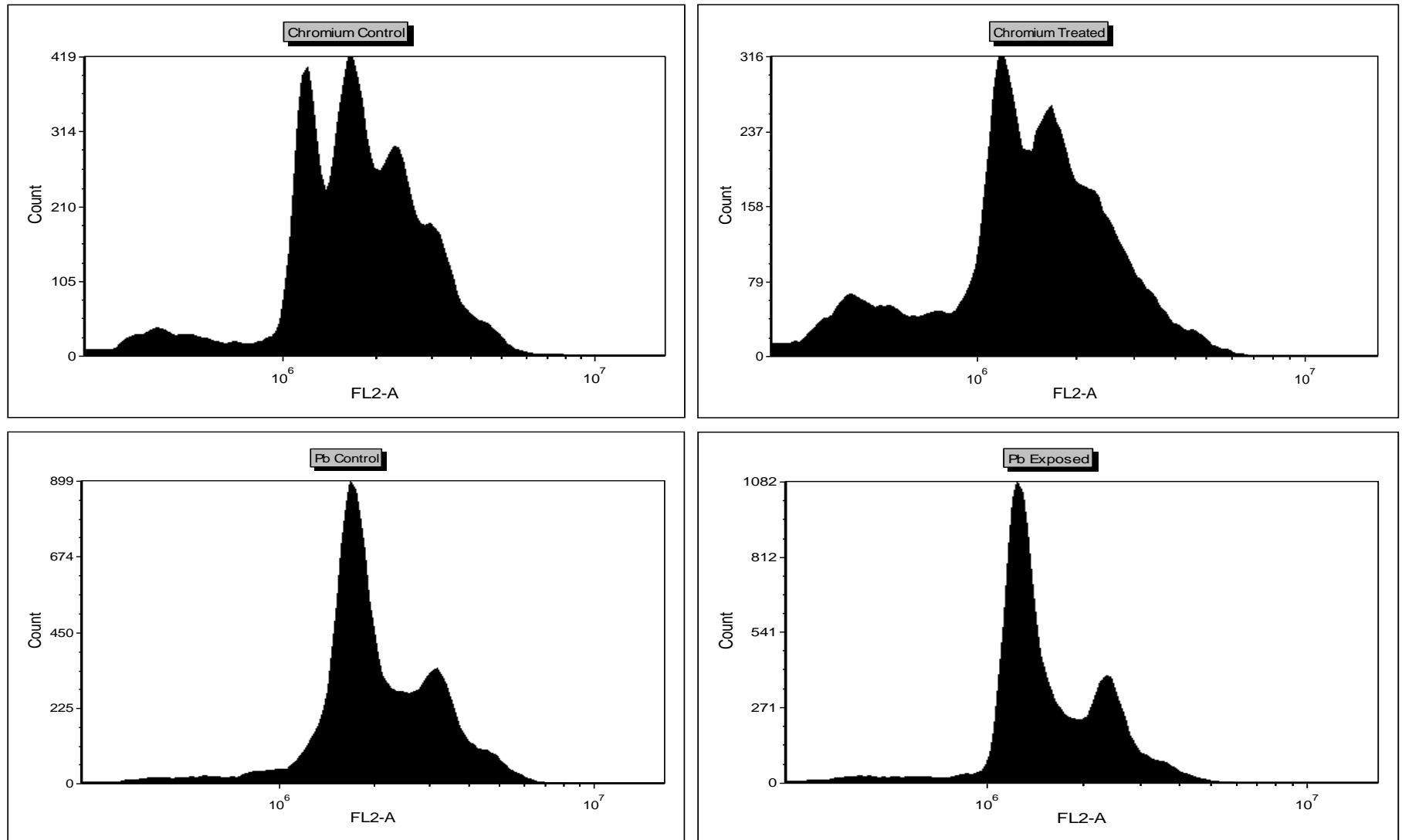


Figure 7. Continued



## CONCLUSIONS

The interactions and cytotoxic effect of selected toxic HMIs (As (III), Cd (II), Cr (VI), Hg (II), and Pb (II)) on human triple negative breast cancer (TNBC) (HCC 1806) was investigated using light microscopy, cell viability, and flow cytometry assays. The results of the study indicated that the influence of HMIs on TNBC cell viability is highly dependent on the type of HMI, and duration of TNBC cell exposure to the selected HMIs. The TNBC cell deaths varied significantly with a maximum of 77% for As (III)-treated TNBC cells to a minimum of 9% cell death for Pb(II)-treated TNBC cells were obtained from the study. The results of the study also suggested that As (III), Hg (II), and Cr (VI) exposures initiate the activation of several cellular and molecular processes including induction of cell death in TNBC cells. Although the molecular mechanism of HMI-induced cell death in cancer cells remains undefined, the current study demonstrates that As (III), Hg (II), Cd (II), and Cr (VI) induced high to moderate cytotoxicity, while Pb (II) had very little to no cytotoxic effect on human TNBC cells.

Differences in the obtained HMIs treated TNBC cell viability may be attributed to differences in the ionic mobility of metal ions across the cell membrane. Transportation of HMIs across cell-membrane may be facilitated by various factors including active transport and ionic diffusion. A heavy metal ion with relatively smaller mass-to-charge ratio is therefore expected to travel faster across the TNBC cell membrane into the cytoplasm. The faster a HMI is able to cross the cell membrane, the quicker the ion can interact with cellular components and organelles such as the mitochondria, ribosome, Golgi body etc. in the cytoplasm, causing cytotoxic effects on TNBC cells. Differences in the affinity and binding capability of HMIs with cellular components, organelle, and nucleic materials may also affect the extent of HMIs cell

viability on TNBC cells. For instance, As (III) and Hg (II) have strong affinities for sulfur. Cellular materials; particularly proteins, are composed of cysteine which contains sulfur. This may explain a comparatively larger cell death obtained for As (III) and Hg (II) treated TNBC cells.

More work is required to gain a better insight into the mechanism of cytotoxic effects of As (III), Hg (II), Cd (II), Cr (VI), and Pb (II) on TNBC cell viability and changes in the cell cycle profiles as revealed in the flow cytometric analysis. There is an ongoing study investigating this mechanism in our research group, and we will determine whether the cell death is attributed to apoptosis and/or necrosis. In addition, this study will be repeated using a normal cell line model as a comparative cell viability analysis.

## Conflict of interest statement

None declared.

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