Underlying mechanisms of cytotoxicity in HepG2 hepatocarcinoma cells exposed to arsenic, cadmium and mercury individually and in combination

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Highlights

- Arsenic, cadmium and mercury was more cytotoxic in combination than individually.
- Mitochondrial membrane depolarisation and redox-status alterations occurred.
- Cell death may occur via necrosis or apoptosis.

Abbreviations: $\Delta\Psi m$: mitochondrial membrane potential; ATP: adenosine triphosphate; As: arsenic; Cd: cadmium; DMSO: dimethyl sulfoxide; EPA: Environmental Protection Agency; GSH: reduced glutathione; Hg: mercury; MCL: maximum contamination limit; PBS: phosphate-buffered saline; ROS: reactive oxygen species.

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Abstract

Background: Toxicity data regarding combinational exposure of humans to arsenic, cadmium and mercury is scarce. Although hepatotoxicity has been reported, limited information is available on their mechanistic underpinnings. The cytotoxic mechanisms of these metals were determined in HepG2 hepatocarcinoma cell lines after individual and combinational exposure.

Methods: HepG2 cells were exposed to heavy metals (sodium arsenite, cadmium chloride, and mercury chloride) individually or in combination for 24 h, after which cell density, mitochondrial membrane potential ($\Delta\Psi m$), reactive oxygen species (ROS), reduced glutathione (GSH), adenosine triphosphate (ATP) and caspase-3/7 activity was assessed.

Results and Discussion: Cadmium (IC_{50} = 0.43 mg/L) and the combination (0.45 mg/L, arsenic reference) were most cytotoxic, followed by arsenic (6.71 mg/L) and mercury (28.23 mg/L). Depolarisation of the $\Delta\Psi m$ and reductions in ROS, GSH and ATP levels occurred. Arsenic, cadmium and the combination increased caspase-3/7 activity, while mercury reduced it.

Conclusion: The combination produced greater, albeit mechanistically similar, cytotoxicity compared to individual metals. Cytotoxicity was dependent on altered mitochondrial integrity, redox-status, and bioenergetics. Although the combination’s cytotoxicity was associated with caspase-3/7 activity, this was not true for mercury. Heavy metal interactions should be assessed to elucidate molecular underpinnings of cytotoxicity.

Keywords: arsenic, cadmium, combination, heavy metals, hepatotoxicity, mercury.

1. Introduction

Heavy metals are generally non-biodegradable and thus tend to persist within the environment.[1,2] Heavy metals, primarily via food and water,[3] is linked to several pathological conditions' onset and progression, including cancer, hepatotoxicity, nephrotoxicity, and neurotoxicity.[1,2] Arsenic (As), cadmium (Cd) and mercury (Hg) have been listed by the Agency of Toxic Substances and Disease Registry as among the top eight most hazardous contaminants in site frequency count.[3] The United States Environmental Protection Agency (EPA) has set maximum contaminant levels (MCL) in drinking water of 10, 5 and 2 µg/L for As, Cd and Hg, respectively.[4]

Global estimates suggest that 160 million people live in areas with increased As levels in drinking water due to enriched geological formations.[5] Although As is a known carcinogen,[6] it is also associated with non-cancerous diseases via the induction of oxidative stress.[7,8] Acute and chronic toxicity results in, among others, cardiovascular (altered haem metabolism, arteriosclerosis, hypertension, ischaemia), hepatic (cirrhosis, hepatomegaly), immune (bone marrow depression, rashes), nephrotic (proximal tubule degeneration) and neurological (encephalopathy, neuropathy) disorders.[9-11]

Human exposure to Cd occurs mainly via cigarette smoke inhalation and the ingestion of contaminated food and water.[12,13] Environmental contamination with Cd occurs through smelting, nickel-Cd batteries, mining and fertilizers.[14] Cadmium promotes DNA damage and apoptosis by inducing oxidative stress.[15] Furthermore, chronic toxicity may perturb serotoninergic and gabaminergic pathways.[15] Acute Cd toxicity can be fatal; resulting in fulminating hepatitis, hemorrhage, pulmonary oedema, and testicular injury. Chronic

intoxication may alter kidney function, disrupt blood pressure control, suppress the immune system, disturb metabolic processes, and induce osteomalacia and pulmonary disease.[12,16-18]

Mercury is released by the cement industry, burning of fossil fuels, mining, and extraction,[19] as well as dumping of inorganic Hg in areas such as the Amazon River in Brazil, and pit-working of gold mines in countries such as Faroe islands, Indonesia, Peru, Philippines, Tanzania, and New Zealand.[20,21] In 1956, 2 200 people in Japan were afflicted with Minamata disease due to consumption of Hg-contaminated fish and shellfish.[22] Acute ingestion leads to abdominal pain, colic, gastroenteritis, renal failure, nausea, and vomiting.[23] Chronic toxicity may cause anorexia, corrosive damage to the mouth, extensive salivation, tremors of the lips and tongue, and loss of teeth.[23]

Heavy metals are systemic toxicants that induce multiple types of organotoxicity, even at low exposure levels.[24] The liver, as the primary metaboliser of the body, is susceptible to such damage during the detoxification process of xenobiotics, including heavy metals.[25] Heavy metals such as As, Cd, and Hg have all been associated with hepatic insult. Arsenic causes liver cancer,[26] portal tract fibrosis, and elevated hepatic enzyme levels.[27,28] Liver cancer is linked to occupational Cd exposure,[29] as well as dose- and time-dependent hepatocellular degeneration and multifocal necrosis, respectively.[30] In chronic poisoning experiments, inorganic Hg induces severe liver injury, indicated by morphological changes, apoptosis and reduced hepatic function.[31]

Heavy metal toxicity is typically a result of combinational exposure rather than individual compounds.[32,33] There is a paucity of literature regarding the combinational effects of As, Cd, and Hg in hepatocellular systems. As the number of possible combinations is infinite, it poses a great challenge for researchers to address the role of mixed exposure in human health. Understanding how metal mixtures affect health is critical to facilitate treatment.[34-36] Many proteins involved in cell growth, apoptosis, oxidative stress, and inflammation are modulated by metals,[37,38] however, mixtures may induce differential responses compared to single metals.[39-42] For example, As neurotoxicity increases when exposure occurs in the presence of other metals.[43] There is thus clearly a need to investigate the potential combinational effect of heavy metals on cellular systems, such as the liver. This study assessed the cytotoxicity of As, Cd and Hg (in isolation and combination) in HepG2 hepatocarcincoma cells to determine potential mechanisms of toxicity.

2. Materials and methods

2.1. Chemicals and reagents

Sodium arsenite (Merck, Germany), cadmium chloride (Sigma-Aldrich, USA) and mercury chloride (BDH, UK) were prepared in deionised water. BBL™ FTA hemagglutination buffer was obtained from BD (France) and used to prepare phosphate-buffered saline (PBS). Hank's Buffered Salt Solution (HBSS) was purchased from Life Technologies (South Africa). Penicillin (10,000 IU) and streptomycin (10 mg/mL) solutions were obtained from BioWhittaker (Walkersville, USA). Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), ATP Assay Kit MAK135, 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH), camptothecin, 3-[(3-chloamidopropyl)dimethylammonio]-1-propanesulfonate, 2',7'-dihydrodichlorofluorescein diacetate (H_2-DCF-DA), Dulbecco's Modified Eagle's Medium

(DMEM), ethylenediaminetetraacetic acid (EDTA), HEPES, JC-1, monochlorobimane, n-ethylmaleimide, phenylmethylsulfonyl fluoride (PMSF), rotenone, saponin, staurosporine, sulforhodamine B (SRB), trypsin, Tris-base and trypan blue were obtained from Sigma-Aldrich (St. Louis, USA). β-Mercaptoethanol and trichloroacetic acid (TCA) was obtained from Merck Chemicals (Darmstadt, Germany). Foetal calf serum (FCS) was procured from PAA (Pasching, Austria).

2.2. Cell culture and seeding

The HepG2 cell line (HB-8065; American Tissue Culture Collection [ATCC]), although cancerous, is widely used for the determination of hepatotoxicity of samples, however, it is of a cancerous origin. Cells were cultured in 75 cm^2 flasks with DMEM containing 10% (v/v) FCS and 1% (w/v) penicillin/streptomycin in a humidified atmosphere of 5% CO_2 and 37˚C. Medium was changed as needed. Once cultures reached a confluence of 80%, cells were harvested by trypsinization and subsequent centrifugation at 200 x *g* for 5 min. Cells were counted using trypan blue exclusion and diluted to 2×10^5 cells/mL. Cells (100 µL) were pipetted into either clear (spectrophotometric assays) or white (fluorometric assays) 96-well plates to achieve 2×10^4 cells/well. Cells were exposed to 100 µL medium (negative control), positive control (refer to respective experiments), deionised water/medium mixture (1:4) (vehicle control) or heavy metal solutions (individual or combination) prepared in FCS-free medium for 24 h. Individual in-reaction exposure ranges were as follows: As (1-12 mg/L), Cd (0.1-2 mg/L) and Hg (10-35 mg/L). The combination was made according to the EPA MCL ratio of As, Cd and Hg (10:5:2), with As used as a reference at 0.1, 0.5, 1.5, 2 and 4 mg/L in-reaction (Supplementary Table 1).

2.3. Cell density

The effect of heavy metal exposure on cell density was determined using SRB staining of TCA-fixed protein elements.[44] Saponin (1% in-reaction) served as the positive control. Treated cells were fixed with 50 µL TCA (50% [w/v]) overnight at 4˚C. After fixation, plates were washed thrice with tap water, dried and stained with 100 µL SRB solution (0.057% [w/v] in 1% [v/v] acetic acid) for 30 min. Plates were rinsed three times with 100 µL acetic acid (1% [v/v]). Bound dye was solubilised with 200 µL Tris-base solution (10 mM, pH 10.5) for 30 min on a shaker at room temperature. Plates were read spectrophotometrically at 510 nm (reference wavelength 630 nm) using an ELX800 plate reader (Bio-Tek Instruments, Inc). All values were blank-subtracted and expressed as a percentage relative to the negative control. Non-linear regression was used to determine the concentration inhibiting 25% (IC_{25}), 50% (IC_{50}) and 75% (IC_{75}) cell growth. A combination index was determined using CalcuSyn software. All further experimentation was conducted at the IC_{25}, IC_{50}, and IC_{75}.

2.4. Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) was monitored using JC-1 ratiometry between monomer and aggregate forms of the dye.[45] Rotenone (200 nM in-reaction) served as the positive control. After treatment, 20 µL JC-1 dye (200 µM in PBS) was added to cells and incubated for 2 h at 37˚C. The medium was replaced with 100 µL HBSS. Fluorescence was monitored using a Synergy 2 plate reader (Bio-Tek Instruments, Inc.) at a monomer excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}) of 485 nm and 525 nm, respectively, while the aggregate form was monitored at λ_{ex} = 545 nm and λ_{em} = 595 nm. The ratio of monomers to aggregates was calculated, and the $\Delta\Psi m$ expressed as a percentage relative to the negative control.

2.5. Intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured using the H_2-DCF-DA cleavage and activation assay.[46] The free radical generator, AAPH (1 mM in-reaction), served as the positive control. After treatment, 20 µL H_2-DCF-DA (110 µM in PBS) was added to cells and incubated for 30 min at 37˚C. The medium was replaced with 100 µL HBSS. Fluorescence was measured at λ_{ex} = 485 nm and λ_{em} = 535 nm. The SRB assay was used to normalise fluorescence to cell density. All values were blank-subtracted and the ROS expressed as a percentage relative to the negative control.

2.6. Intracellular reduced glutathione

Intracellular reduced glutathione (GSH) levels were monitored using the monochlorobimane adduct formation assay.[47] n-Ethylmaleimide (25 µM in-reaction) served as the positive control. After treatment, 20 µL monochlorobimane (176 µM in PBS) was added to cells and incubated for 2 h at 37˚C. The medium was replaced with 100 µL HBSS. Fluorescence was measured at λ_{ex} = 360 nm and λ_{em} = 460 nm. The SRB assay was performed after measurement to normalise fluorescence to cell density. All values were blank-subtracted and the reduced glutathione expressed as a percentage relative to the negative control.

2.7. Intracellular adenosine triphosphate

Adenosine triphosphate (ATP) levels were determined using chemiluminometry as a measure of cell viability according to the ATP Assay Kit MAK135 manufacturer's instructions.[48] Staurosporine (10 µM in-reaction) and saponin (1% in-reaction) served as apoptotic and necrotic positive controls, respectively. The medium was replaced with 90 µL ATP reagent and the plate incubated for 1 min. Luminescence was read using a

chemiluminescence plate reader (Synergy 2, Bio-Tek Instruments, Inc). Luminescence was normalised to the average cell density of the respective treatments, and the ATP expressed as a percentage relative to the negative control.

2.8. Caspase-3/7 activity

Caspase-3/7 activity was measured using Ac-DEVD-AMC cleavage as an indication of caspase-dependent apoptosis.[49] Camptothecin (40 µM in-reaction) served as the positive control. The medium was replaced with 25 µL cold lysis buffer (containing 10 mM HEPES, 2 mM CHAPS, 5 mM EDTA, 0.5 mM PMSF, and 4.3 mM β-mercaptoethanol [the latter two added 30 min before use]) and incubated for 15 min on ice. After lysis, 100 µL caspase-3/7 substrate buffer (containing 20 mM HEPES, 2 mM EDTA, 0.5 mM PMSF, 4.3 mM β-mercaptoethanol and 5 µM Ac-DEVD-AMC [the latter three added 30 min before use]) was added and plates incubated for 4 h at 37˚C. Fluorescence was measured at λ_{ex} = 355 nm and λ_{em} = 460 nm. Cell density was determined in alternative wells by the SRB assay. Fluorescence was normalised to the average cell density of the respective treatments, and the caspase-3/7 activity expressed as a percentage relative to the negative control.

2.9. Statistics

All experiments were conducted with a minimum of three technical and three biological replicates. All results were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were done using GraphPad Prism 5.0. The inhibitory concentrations were determined using non-linear regression. Differences between the negative control and samples were determined using Kruskal-Wallis with a post-hoc Dunn's test. Significance was deemed as $P<0.05$.

3. Results

3.1. Cell density

Of the three heavy metals tested, Cd was the most cytotoxic, followed by As and Hg (Table 1). The cytotoxicity of the combination of heavy metals was in the same concentration range (with As used as reference) as Cd.

Table 1: Inhibitory concentrations of As, Cd and Hg after 24 h exposure in the HepG2 cell line.

Inhibitory concentration	Arsenic	Cadmium	Mercury	Combination[a]
IC_{25} (mg/L) ± SEM	4.01 ± 1.24	0.22 ± 2.00	22.90 ± 1.18	0.32 ± 1.11
IC_{50} (mg/L) ± SEM	6.71 ± 1.14	0.43 ± 1.40	26.23 ± 1.06	0.45 ± 1.35
IC_{75} (mg/L) ± SEM	9.61 ± 1.08	1.11 ± 2.33	33.04 ± 1.05	1.63 ± 1.12

[a]The As concentration is taken as reference, however, comprises all three metals.

Arsenic decreased cell density significantly ($P<0.05$) at ≥6 mg/L, with a maximum decrease of 86% at 12 mg/L (Figure 1A). Cell density was decreased significantly ($P<0.05$) by Cd at ≥0.25mg/L, with up to 86% reduction at 2 mg/L (Figure 1B). Mercury reduced cell density by 79% at 35 mg/L, though significant ($P<0.05$) reductions were apparent from ≥20 mg/L (Figure 1C). The combination from 0.5 to 4 mg/L As reference concentration decreased cell density by 45 to 93%, however, the lowest concentration of the combination (0.1 mg/L) did not reduce cell density (Figure 1D). At the IC_{50} of the combination (As = 0.45 mg/L; Cd = 0.23 mg/L; Hg = 0.09 mg/L), a combination index of 0.80366 was calculated, suggesting synergistic activity.

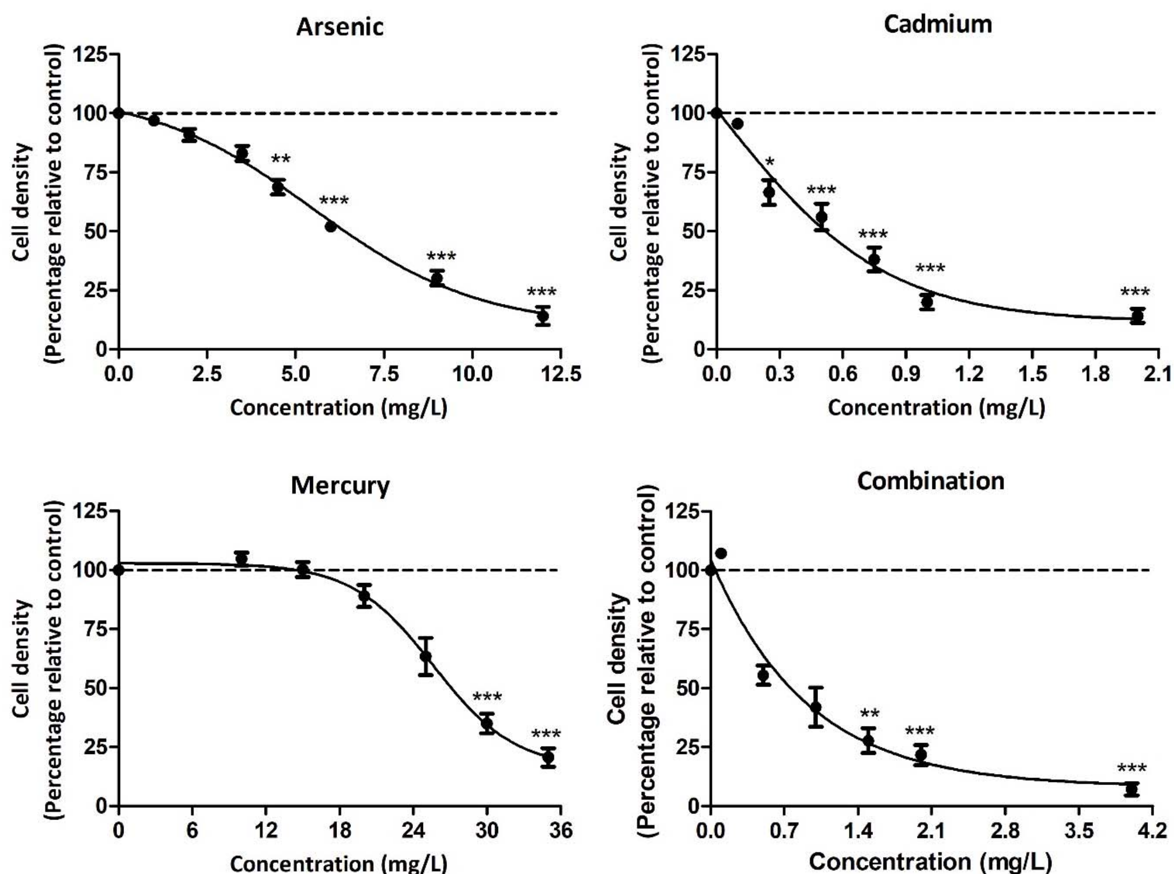

Figure 1: Dose-dependent reduction of cell density after exposure to As, Cd, Hg and the combination for 24 h. Significance in comparison to the negative control: *** P<0.001, ** P<0.01, * P<0.05.

3.2. Mitochondrial membrane potential

At their respective inhibitory concentrations, depolarisation was most prominent after exposure to Cd, followed by Hg and As. Arsenic displayed a plateaued reduction of ΔΨm at the IC_{25} and IC_{50} (~12%), however, a significant (P<0.05) reduction of 45% was observed at the IC_{75} (Figure 2A). Cadmium dose-dependently decreased ΔΨm by 16%, 39% and 74% at the IC_{25}, IC_{50} and IC_{75}, respectively (Figure 2A). Mercury displayed a plateaued reduction at the IC_{25} and IC_{50} of 29% and 33%, respectively, followed by a 60% reduction at the IC_{75} (Figure 2A). The combination treatment decreased ΔΨm significantly (P<0.05) from 1.5 mg/L As reference concentration by 54%, with a maximum reduction of 78% at 4 mg/L (Figure 2B).

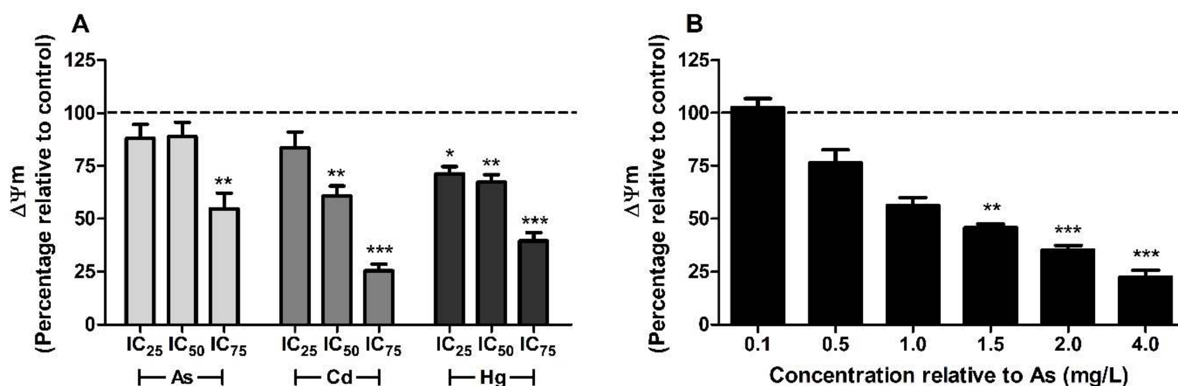

Figure 2: Depolarisation of the mitochondrial membrane after 24 h exposure to (A) individual metals and (B) the combination. Significance in comparison to the negative control: *** $P<0.001$, ** $P<0.01$, * $P<0.05$

3.3. Reactive oxygen species

All heavy metals decreased ROS levels. The reduction was most evident after As exposure, followed by Hg and Cd. Arsenic reduced ROS by 28%, 44% and 57% at the IC_{25}, IC_{50} and IC_{75}, respectively (Figure 3A). A similar trend was observed for Cd and Hg, where Cd reduced ROS levels by 11%, 28% and 45% at respective inhibitory concentrations (Figure 3A). Mercury did not decrease ROS at the IC_{25}, but reduced it by 51% at the IC_{75} (Figure 3A). The combination treatment at 1.5 to 4 mg/L As reference concentration decreased ROS significantly ($P<0.05$) by 77.14% to 84.65%, though no effect was present at 0.1 mg/L (Figure 3B).

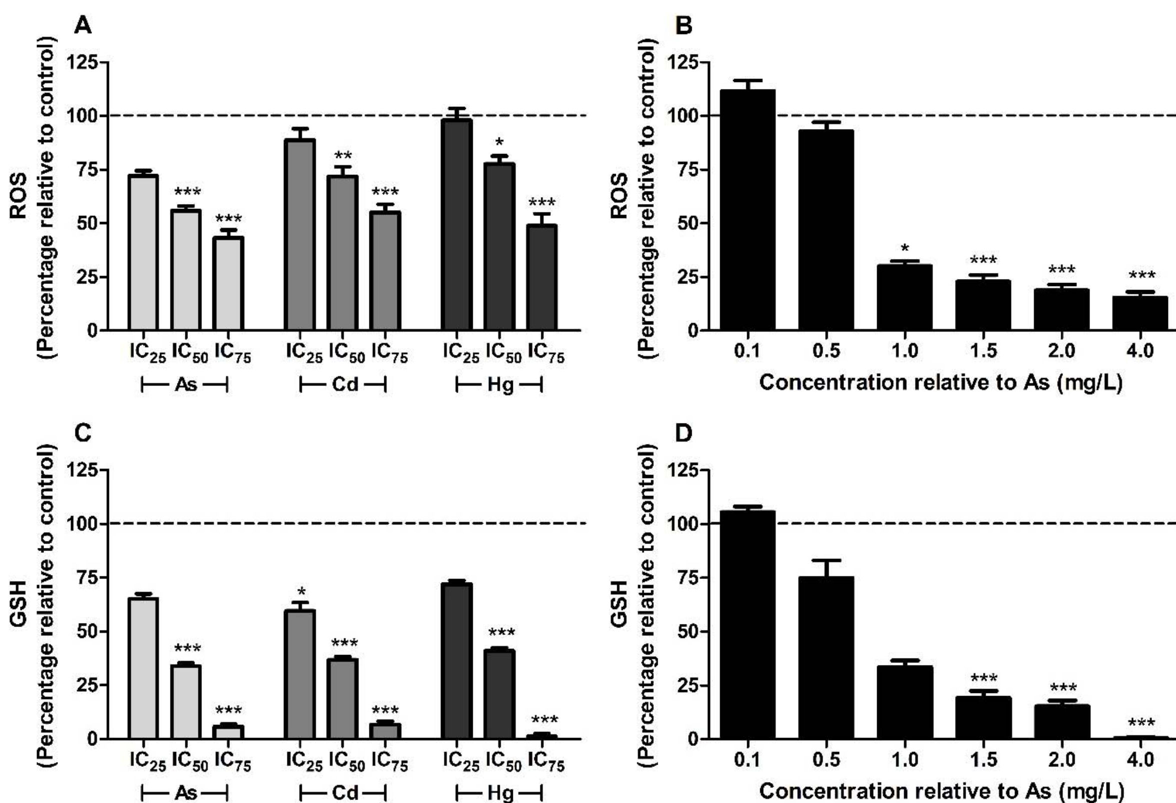

Figure 3: Decreased (A,B) ROS and (C,D) GSH levels after 24 h exposure to (A,C) individual metals and (B,D) the combination. Significance in comparison to the negative control: *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

3.4. Reduced glutathione

All heavy metals decreased GSH levels to a similar extent. Arsenic decreased GSH by 35%, 64% and 94% at the IC_{25}, IC_{50} and IC_{75}, respectively (Figure 3C). The IC_{25}, IC_{50} and IC_{75} of Cd decreased GSH by 40%, 60% and 93%, respectively (Figure 3C). A reduction of 28%, 59% and 99% was observed at the IC_{25}, IC_{50} and IC_{75} of Hg, respectively (Figure 3C). The combination reduced GSH by 25% to 99% at 0.5 to 4 mg/L As reference concentration, however, no effect was observed at 0.1 mg/L (Figure 3D).

3.5. Adenosine triphosphate

Mercury decreased ATP the most, followed by As and Cd. Arsenic reduced ATP dose-dependently at the IC_{25} (68%), IC_{50} (77%) and IC_{75} (87%) (Figure 4A). Cadmium had a less pronounced reduction at the IC_{25} (39%), and a maximum reduction of 88% at the IC_{75} (Figure 4A). Adenosine triphosphate was decreased by 86%, 90% and 100% at the IC_{25}, IC_{50} and IC_{75} of Hg, respectively (Figure 4A). The combination treatment at 1 to 4 mg/L As reference concentration decreased ATP by 20 to 93%, however, no decrease was observed at lower concentrations (0.1 and 0.5 mg/L) (Figure 4B).

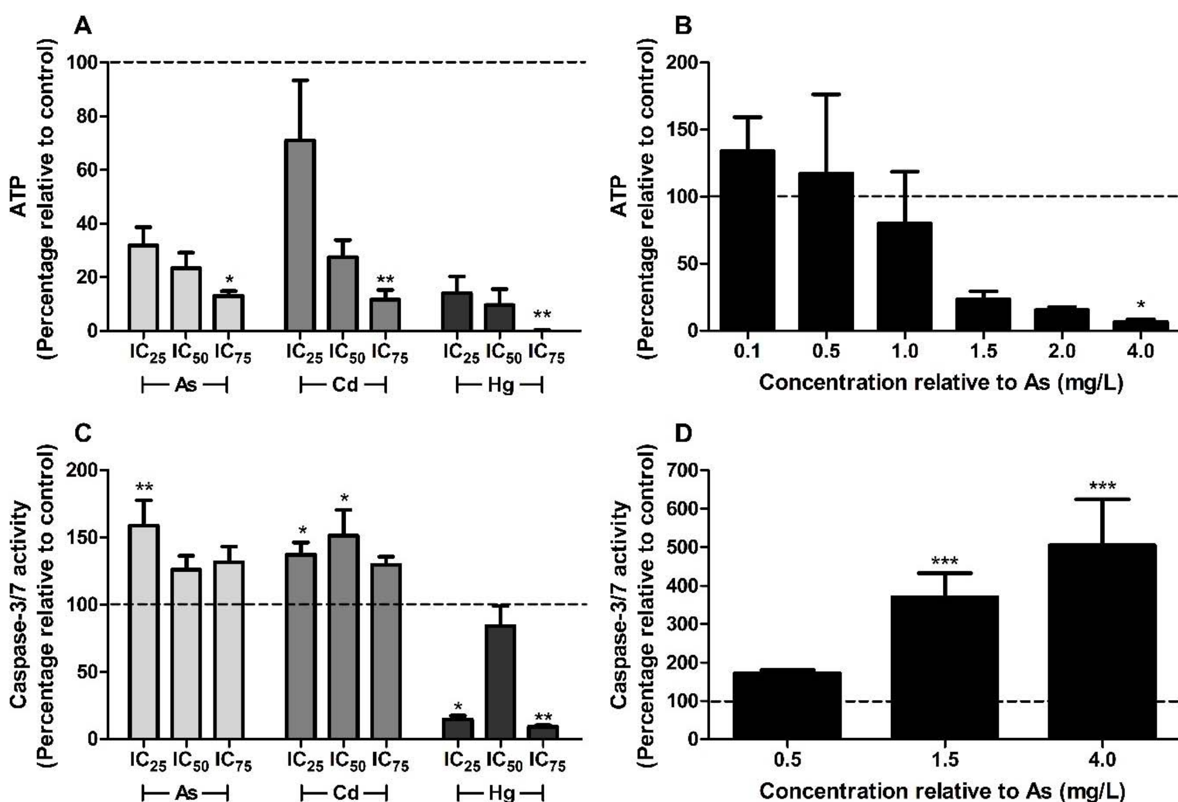

Figure 4: Alteration to (A,B) ATP and (C,D) caspase-3/7 activity after 24 h exposure to (A,C) individual metals and (B,D) the combination. Significance in comparison to the negative control: *** P<0.001, ** P<0.01, * P<0.05.

3.6. Caspase 3/7 activity

Arsenic and Cd activated caspase-3/7, however this was not dose-dependent, whereas Hg decreased it to below the baseline. For As a plateaued increase in caspase-3/7 activity was evident (between 126% and 159%) with the IC_{25} producing the most pronounced effect (Figure 4C). Similarly, Cd increased caspase-3/7 activity by between 130% and 151% (Figure 4C). Mercury decreased caspase-3/7 activity by 85% when treated with the IC_{25} and IC_{75}, however, only by 16% at the IC_{50} (Figure 4C). The combination treatment at 0.5, 1.5 and 4 mg/L As reference concentration increased caspase-3/7 activity dose-dependently by 172%, 370% and 505%, respectively (Figure 4D).

4. Discussion

Differential effects were observed between the individual metals and the combination thereof. As there is a paucity of recent literature regarding the effect of the heavy metals on human primary hepatocytes, comparisons are made to published literature using immortalise cell ines. In the present study, As (as arsenite) reduced cell density by approximately 60% at 7.4 mg/L, however, higher concentrations (25.98 mg/L) were necessary to accomplish this in Chang human hepatocytes.[50] Arsenite displayed an IC_{50} of 8.4 mg/L in human primary hepatocytes,[51] which is in accordance to the IC_{50} obtained in the present study. The IC_{50} of arsenite in rat heart micro-vessel endothelial cells (4.6 mg/L)[52] was similar to that calculated in the present study (6.71 mg/L). Uptake of As differs between cell lines,[53] and thus contributes to the variance obtained between studies. Furthermore, the oxidation state of As affects cytotoxicity, with arsenite being more potent than arsenate.[52] Significant mitochondrial depolarisation was only observed at the IC_{75}, suggesting that low levels of depolarisation may be sufficient to promote cytotoxicity. Di-arsenic trioxide (1 to 5 mg/L)[54] and sodium

arsenite (2.6 to 3.9 mg/L)[55] dissipated the ΔΨm in HepG2 and Chang cells, respectively. Sodium arsenite-induced mitochondrial membrane depolarisation facilitates apoptosis via cytochrome c release,[55] however, mitochondria-independent apoptosis has also been described in HepG2 cells.[56] Caspase-3 can be activated by the death receptor pathway, which is not reliant on mitochondrial perturbations,[56] and ultimately leads to apoptosis.[57] Caspase-3/7 activity has been reported in hepatocellular carcinoma cells as low as 0.1 mg/L di-arsenic trioxide As_2O_3,[57] while sodium arsenite at 3.9 mg/L induced apoptosis in human epidermoid carcinoma A431 cells and EA.hy926 cells.[58] Corroborating the cell death observed, ATP levels were also reduced at low concentrations. Similar effects have been described in wild-type mouse embryonic fibroblasts cells exposed to 2 mg/L As_2O_3.[59] Contrary to what is expected, mitochondrial depolarisation, however slight, did not increase ROS but rather decreased it. Arsenic has been shown to generate ROS in Chang cells,[55] ascites hepatoma AS-30D cells[60] and male Dunkin–Hartley guinea pigs.[61] The ROS depletion may be indicative thereof that reductive stress occurred. Of importance, the H_2-DCF-DA assay, which is widely used to determine ROS, is known to have several limitations, such as being subject to interference from other co-factors, and thus further analysis is needed to ensure results are a true reflection of the biological occurrence.[62] Kinetic evaluation would be beneficial to showcase ROS levels throughout the exposure time-frame, and not just a cross-sectional effect thereof. Furthermore, GSH was reduced dose-dependently by As, which is supported in literature.[63] Such an effect is likely due to conjugation reactions, as trivalent arsenicals form complexes with thiol-containing molecules, such as GSH and cysteine, *in vitro.*[64]

Cadmium was the most cytotoxic heavy metal tested, producing detriments at lower concentrations than the other metals. Cytotoxicity has been reported, albeit at higher concentrations (IC_{50} ranging between 1.1 and 1.6 mg/L in HepG2 cells).[65] The gradual mitochondrial membrane depolarisation, paralleling reduced cell density and ATP levels with increased caspase-3/7 activity, is suggestive of mitochondrial-dependent apoptosis. Mitochondrial depolarisation is known to increase the release of pro-apoptotic mediators.[66,67] Cadmium chloride is reported to dissipate $\Delta\Psi m$ at high concentrations (18.3 mg/L) in normal human hepatocytes (NHH) and SV40-immortalized human hepatocytes (IHH),[68] which may interrupt bio-energetic production of ATP,[69] as was observed in the present study. Such ATP depletory effects have been described for Cd in HepG2, human astrocytoma 132INI and human embryonic kidney HEK293 cells.[69] Furthermore, Cd is pro-apoptotic in the liver,[70] with its chloride form (0.9 mg/L to 1.8 mg/L) activating caspase-9 in C6 glioma cells.[71] Although Cd has been described to generate ROS,[60,69,72] it has also been shown to decrease after the initial increase,[60,72] suggesting that the free radical response may have been missed at the 24 h assay point. Literature corroborates GSH depletion, possibly due to the inhibition of glutathione reductase.[73]

Mercury was the least cytotoxic of the three heavy metals tested. Mercury-induced cytotoxicity has been described in several cell lines, including AS-30D rat hepatoma cells.[72] The differences observed in the intensity of cytotoxicity appears to be dependent on the cell line, experimental end-point, and Hg's oxidation state. As with Cd, Hg gradually depolarised the mitochondrial membrane; similar effects have been reported for methyl mercury in PC12 cells[74] and astrocytes.[75] The depolarisation in astrocytes was induced by lower concentrations of Hg, which may be ascribed to the organic form of Hg used.[75] The

mitochondrial effects were paralleled by a large decrease in ATP, however, caspase-3/7 activity also decreased. The latter is suggestive thereof that a non-caspase-dependent route of cytotoxicity, which is contradicted to what has been described in differentiated neuronal WHMES cells[76] and human lymphocytes[77] at higher concentrations, is followed. Reduced ATP levels have also been reported after exposing isolated perfused rat liver to Hg.[78] Excessive ATP depletion can transition cell death from apoptosis to necrosis,[79,80] which may explain the lack of caspase-3/7 activity. Although mitochondrial depolarisation was observed, ROS levels decreased. Similar effects have been reported after Hg exposure in PC12 and AS-30D cells due to an inhibition of cellular respiration.[72,74] Although short incubation periods would appear to increase ROS generation (<24 h), reductions are apparent at later time-points.[81] As in the present study, literature supports GSH reductions in HepG2 cells[82] and A172 human glioma cells,[83] however, at higher concentrations than what was used in the present study. This effect is likely due to detoxification, as GSH binds covalently to Hg to expel it from cells, though exaperates its cytotoxicity.[84]

Combinations of As, Cd, Hg and lead are known to be more cytotoxic than individual metal exposures. In the present study, a 14.9-fold increase in cytotoxicity was noted in comparison to As's IC_{50}, indicating a potentiation of its activity. The IC_{50} of As was decreased in an EPA combination of Cd, Hg and Pb to 0.62 mg/L when compared to its individual IC_{50} (1.6 mg/L).[85] To the authors' knowledge, no literature is available to describe the effects of such combinations on $\Delta\Psi m$, ATP, ROS, GSH and caspase 3/7 activity in HepG2 cells. In the present study, mitochondrial depolarisation was observed, with associated reductions in GSH and ATP, and increased caspase-3/7 activity. As with the individual metal exposures, ROS was reduced, which may be indicative that reductive stress occurred. According to the

combination index, a synergistic potential is present, which aligns to the differential drug targets of each heavy metal as a contributing factor to cytotoxicity. Based on observations, it is likely that a loss of mitochondrial integrity[66,67] and perturbations of the redox status activated pro-apoptotic factors,[58,70,76] leading to cell death.

5. Conclusion

Both individual and combination exposure of the metals were cytotoxic to HepG2 hepatocarcinoma cells, however, the combination produced cytotoxicity at much lower concentrations. Mitochondrial depolarisation, to various degrees, was associated with all exposures. Except for Hg, caspase-3/7 activity was induced for all metals and the combination, suggesting an apoptotic route of cell death, however, its dependency on mitochondrial effects is unclear. Additionally, all metals and the combination decreased ATP and GSH levels. The reduced ROS levels may indicate an extent of reductive stress being incurred in the cell line. As metal exposure rarely occurs in isolation, combinational effects should be assessed do elucidate the molecular underpinnings of heavy metal cytotoxicity, which may expose potential treatment options and diagnostic criteria for associated hepatotoxicity.

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