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4-Methylcyclohexane methanol (MCHM) affects viability, development, and movement of *Xenopus* embryos

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ABSTRACT

Following chemical spill disasters, it is important to estimate the effects of spilled chemicals on humans and the environment. Here we analyzed the toxicological effects of the coal cleaning chemical, 4-methylcyclohexane methanol (MCHM), which was spilled into the Elk River water supply in 2014. The viability of HEK293 T human cell line cultures and *Xenopus tropicalis* embryos was negatively affected, and the addition of the antioxidants alleviated toxicity with MCHM exposure. Additionally, *X. tropicalis* embryos suffered developmental defects as well as reversible non-responsiveness and melanization defects. The impact MCHM has on HEK293 T cells and *X. tropicalis* points to the importance of continued follow-up studies of this chemical.

1. Introduction

The chemical 4-methylcyclohexane methanol (MCHM) was a previously little-studied chemical involved in the processing of coal, until a rusted storage tank resulted in a spill of crude MCHM mixed with stripped PPH (propylene glycol phenyl ether and dipropylene glycol phenyl ether) into the Elk River near Charleston, WV in 2014. The spill's size and location adjacent to a drinking water treatment intake were sufficient to fill homes with an overpowering odor that left many fearful of potential negative health consequences. The concentration of MCHM was highest, greater than 100 ppm, at the Kanawaha valley water treatment plant intake and detectable amounts, 5.5 ppb, reached as far as 350 miles away in the Ohio river [1,2]. MCHM also entered households at concentrations as high as 10−420 ppb [3]. This spill interrupted the water supply of approximately 300,000 residents [4]. Research on MCHM since the Elk River spill has increased dramatically over concerns about the lack of characterized physical and biological properties of this chemical [5]. Children born from mothers pregnant during the spill did not have significant health effects [6]. This could be due to a no use order given to pregnant women resulting in a lack of exposure that could account for a change in APGAR scores or due to no effect of the chemical on fetal development [6]. Studies on zebrafish at levels of the MCHM mixture that was present at the water intake for the Kanawha Valley Water Treatment plant prevented eggs from hatching [7], while effects from exposure to the crude MCHM was less. The chemicals in the MCHM mixture also have a synergetic effect on the viability of bacteria and human cells in tissue culture compared to the crude MCHM [8,9]. However, in yeast, no change in growth was noted between the MCHM mixture and the crude MCHM (C. Nassif and J. Gallagher, unpublished). An improved toxicological study on MCHM's effect on model organism viability has been performed [10], as well as studies on the potential stress responses it may produce in yeast [11–14]. From the transcriptional analysis, diverse pathways are affected by crude MCHM [13,14]. MCHM acts as a hydrotrope, altering the protein structure and changing the intercellular concentrations of metals [13], and long-term exposure increases internal levels of Reactive Oxygen Species (ROS) [14].

ROS has previously been implicated as a source of toxicity in cells treated with MCHM [11,14,15]. Cells contain conserved robust networks to mitigate the toxic effects of ROS. These include various proteins, from enzymes that detoxify the reactive species directly, to proteins that repair damage within the cell, such as to DNA [16]. The thioredoxin and glutathione (GSH) pathways have significant roles in the cell's response to ROS. They perform overlapping functions mitigating thiol-reduction issues that can damage proteins in the cytosol. Furthermore, GSH has roles in iron homeostasis between the mitochondria and vacuole, and potentially as a possible buffer for oxidation

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in disulfide bond formation during protein folding in the ER [17,18]. These pathways are activated by normal metabolic processes as the cell meets its normal energy needs through respiration. However, they also become important during the response to toxic chemicals, which often produce ROS directly, or else indirectly through metabolism and attempted detoxification in the vacuole. Toxicity of chemicals that produce ROS in the cell may be mitigated through treatment with antioxidants or intensified through damage to the cellular stress networks [19,20]. Exposure to metals such as unchaperoned copper, an essential trace mineral, induces ROS that affects the mitochondria and cell lipids [21,22]. When cotreated with GSH, the capacity of cells to tolerate higher internal copper levels increases, and viability is rescued [21].

MCHM acts as a hydrotrope *in vitro*, changing the solubility and protein structure [13]. MCHM changes intracellular levels of a wide range of ions including calcium [13]. The metabolome is also altered including increased levels of every amino acid detected [12]. The molecular target of MCHM is unknown but biologically active hydrotropes change the liquid-liquid phase separation of transcription factors and RNA binding proteins [23–26]. In yeast, variation in the length of a polyglutamine repeat in a protein involved in transcription regulated the response to MCHM [14]. The wide range of effects was also reflected in the transcriptomics [12,13]. Numerous stress and biosynthetic pathways were both down and upregulated in responses to MCHM. Extended exposure to MCHM also increases internal ROS levels [14]. Petite yeast (yeast without functioning mitochondria) have higher levels of endogenous ROS and are more sensitive to MCHM [13].

Xenopus tropicalis is an excellent model organism for studying developmental toxicity from chemical spills. *X. tropicalis* is high throughput, laying thousands of embryos from a single mated pair, and data generated in *X. tropicalis* may be extrapolated to other species with an accuracy greater than 85 % [27]. *X. tropicalis* also has the added advantage of being directly applicable to aquatic organisms affected by the MCHM spill. When *X. tropicalis* embryos were exposed to levels comparable to the crude MCHM levels present in the water during the spill, their viability decreased. There were also physical aberrations including an underdeveloped gastrointestinal tract, increased

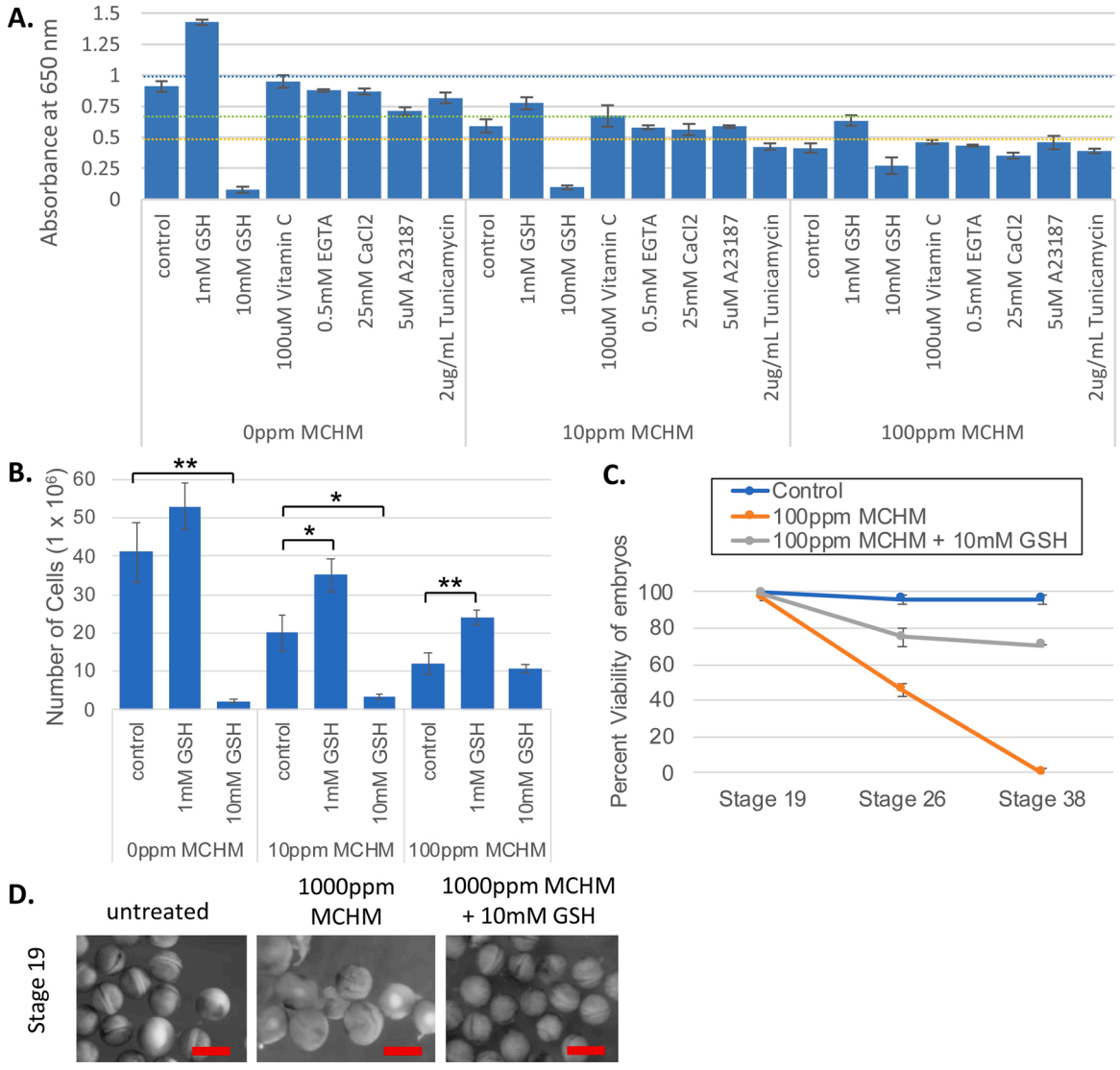

Fig. 1. ***Effects of MCHM on human HEK293 T cells and Xenopus tropicalis embryos viability.*** **A.** Viability of HEK293 T cells as indicated by the MTT assay treated with an increasing amount of MCHM together with various chemicals indicated in the graph. The average and standard error are graphed. Viability in control at 0 ppm of MCHM is marked with a blue dashed line. Viability in control at 10 ppm of MCHM is marked with a green dashed line. Viability in control at 100 ppm of MCHM is marked with an orange dashed line (N = 4). B. The number of HEK293 T cells with an increasing amount of MCHM supplemented with 1 mM or 10 mM GSH. **C.** *X. tropicalis* embryos were treated with 100 ppm MCHM or 100 ppm MCHM supplemented with 10 mM GSH at NF stage 19 (n = 400 per sample, N = 4). **D.** Stage 19 embryos untreated, treated with 1000 ppm MCHM, or 1000 ppm MCHM supplemented with 10 mM GSH starting at NF stage 2 (n = 300 per sample, N = 3), the red scale bar is 2 mm; * $p \leq 0.05$; ** $p \leq 0.01$.

melanocyte migration and/or size, and a non-responsive phenotype denoted by not moving when shaken or poked. While the mechanism for non-responsiveness in the tadpoles is unknown, the kinetics of non-responsiveness parallels the effect of sodium channel inhibitors benzocaine and lidocaine and the acetylcholine receptor agonist on the tadpoles [28].

In this paper, we show that MCHM impacts the viability of human cells and vertebrate organisms that can be rescued in part with GSH. We further show that at low concentrations, MCHM has a non-responsive effect on vertebrate organisms similar to vgNa channel inhibitors lidocaine and benzocaine. This points to a potentially important effect that could inform unexpected toxic effects in humans and other animals. In this way, these data can inform decisions where previous knowledge from basic toxicological studies of this chemical is lacking.

2. Results

The viability and phenotypic effects of crude MCHM on metazoans were tested. The viability of human embryonic kidney HEK293T cell line was tested *via* an MTT assay and then supplemented with various chemicals to determine if any could mitigate the effects of MCHM. When MCHM was not present the addition of 1 mM glutathione (GSH) increased viability by 57 %, indicating that the growth media is not optimal for these cell lines (p-value = 2.47 × 10−5, Fig. 1A). At 10 mM GSH, viability decreased almost 12-fold (p-value = 4.15 × 10-6). Vitamin C (ascorbic acid), another potent antioxidant, did not alter viability (p-value = 0.19). The addition of a divalent chelators EGTA also had little effect on viability (p-value = 0.14). The levels of calcium were altered by two different mechanisms, the addition of 25 mM $CaCl_2$ or 5 μM A23187, a calcium ionophore that increased the intracellular levels of Ca^{2+}. A23187 decreased viability by 12 % (p-value = 0.0014), while $CaCl_2$ did not alter viability (p-value = 0.115). Tunicamycin induces the unfolded protein response and MCHM changes the solubility of proteins *in vitro* [13]. There was a 35 % decrease in absorbance when cells were treated with 10 ppm MCHM, indicating a decrease in viability (p-value = 4.93 × 10-5). This was partially rescued by 1 mM GSH (to 85 % of untreated), consistent with our observation above that ROS contributes to the cytotoxic effects of MCHM (Fig. 1A); however, it did not completely restore viability to untreated levels (p-value = 0.00598). 10 ppm and 100 ppm MCHM also greatly reduced the number of HEK293T cells that was significantly increased by supplementing with 1 mM GSH (Fig. 1B). The divalent chelator, CaCl2, and calcium ionophore did not alter response in 10 ppm MCHM (p-values = 0.254, 0405, 0.447, respectively). Tunicamycin further decreased viability in conjunction with 10 ppm MCHM (p-value = 1.181 × 10-4). Treatment with 100 ppm MCHM further decreased viability by 55 % compared to the untreated control (p-value = 5.93 × 10-5). At 100 ppm MCHM, 1 mM GSH improved the growth of the 100 ppm MCHM treated cells. The 1 mM GSH with 100 ppm MCHM viability was no different from that of cells treated with 10 ppm MCHM alone (p-value = 0.168). Interestingly, 10 mM GSH with 100 ppm MCHM improved viability compared to 10 mM GSH alone (p-value 0.00436) or combined with 10 ppm MCHM (p-value = 0.00554). All other additives tested had little to no significant change the viability with 100 ppm MCHM (vitamin C = 0.061, EDTA = 0.22, $CaCl_2$ = 0.0428, A23187 = 0.157, and tunicamycin = 0.201).

To assess the impact of MCHM on a multicellular organism, viability, and development of the Western clawed frog *Xenopus tropicalis* embryos were characterized. 100 *X. tropicalis* embryos were treated with 100 ppm MCHM starting at stage 19 and followed until stage 38 (Fig. 1C). Viability immediately dropped at stage 26–40%, and by stage 38 all the embryos died. The addition of GSH rescued the embryo viability to 82 %. We tested this dose of MCHM (100 ppm) on embryos at NF stage 19. 51 % of the embryos died by stage ~40, as compared to a control group's untreated death rate of 3 % (Fig. 1C). *X. tropicalis* embryos exposed at NF stage 2 to the maximum solubility of MCHM, 1000 ppm, were all apoptotic by stage 19 compared to untreated controls (Fig. 1D). Addition of GSH to *X. tropicalis* embryos which partially rescued viability, also prevented the embryos from swelling and turning white, but interestingly also caused arrested development (Fig. 1D). The viability of *Xenopus* embryos was affected at MCHM concentrations greater than or equal to 100 ppm which was 10-fold more sensitive to MCHM than yeast [14,29].

To examine the effects of low-dosage MCHM on the health at later stages, stage 35 tadpoles were exposed to 0.1−40 ppm MCHM; the container was agitated every five minutes and the tadpoles gently poked (Fig. 2A and Supplemental Fig. 1). In 10 ppm MCHM, within 5 min of treatment 70 % of the tadpoles failed to respond to stimuli, and within 15 min all tadpoles exhibited non-responsiveness. When tadpoles were exposed to only 1 ppm MCHM, non-responsiveness began between 10 and 15 min, and the maximum non-responsiveness occurred within 20 min with 60 % of embryos unresponsive. Beginning at 30 min, non-responsiveness began to decrease until the end of observation at 60 min. The non-responsiveness was reversible because washing out MCHM returned full mobility to the embryos (data not shown). We treated embryos with inositol and GSH and there was no rescue in MCHM non-responsiveness under the same conditions (Supplemental Fig. 1). Because the MCHM non-responsiveness phenotype was similar to the reversible vgNa channel inhibitors, we compared MCHM, benzocaine, and lidocaine side by side (Fig. 2B) [30]. Lidocaine reduced the movement of the tadpoles beginning at a 0.02 ppm and were non-responsive at 20 ppm. Benzocaine was less potent and reduced the movement of the tadpoles at a 1 ppm with non-responsiveness at 10 ppm.

When the embryos were cultured at 100 ppm MCHM shortly after fertilization, many of the embryos exhibited underdeveloped gastrointestinal tracts (Fig. 3A). Beginning at stage 26, the MCHM treated embryos had underdeveloped rostral and caudal ends that continued until stage 40. As a whole, the embryos appeared truncated, indicating an anterior-posterior axis defect. The eye was also underdeveloped and lacked pigment compared to the untreated control embryo. To determine if these morphological changes were dose-responsive, embryos were treated with 0.1, 1, 10, 40, and 100 ppm of MCHM (Fig. 3B). Only at greater than or equal to 40 ppm did MCHM cause underdeveloped gastrointestinal tracts, a smaller body length, and a reduction in melanocyte size, number, or migration. However, at 0.1 ppm–10 ppm MCHM, the melanocytes seemed to expand that appeared to go away at higher concentrations. This may be due to MCHM having pleiotropic effects. MCHM induced phenotypes were not apparent at any concentration of benzocaine nor lidocaine (Fig. 3C-D). However, the expanded shape of the melanocytes was noted in both benzocaine and lidocaine treatment; indicating that one effect MCHM may have is to affect vgNa channels. The pigmentation increase at 1 ppm of MCHM was 43 % which was similar to 2 ppm lidocaine (45 %). Benzocaine was used at a wide dose range and a concentration that would cause approximately a similar increase of pigmentation would be between 1 and 10 ppm. When the embryos were cultured at 40 ppm MCHM shortly after fertilization, many of the embryos underdeveloped gastrointestinal tracts in benzocaine or lidocaine (Fig. 3C-D). To further test the underdeveloped gastrointestinal tract, we used a *snai2-eGFP* transgenic line recently developed to assess the development of the cranial neural crest [31]. Melanocytes are derived from the neural crest and migrate along the dorsolateral axis to their final locations. Snai2 is a transcription factor that is expressed early and is a marker for the cranial neural crest differentiation [31]. While the underdeveloped gastrointestinal tract and increase in melanocyte size and/or migration was still present in MCHM treated embryos, there was no effect on Snai2 driven GFP (Fig. S2).

These severe defects confirm that further study of this chemical in various model organisms would be beneficial. Treatment of yeast gave insight into the cellular pathways affected by MCHM treatment, but the treatment of the embryos and human cell lines revealed that the lack of viability reduction as a phenotype for yeast [15] did not indicate that the chemical itself is relatively safe for humans or the environment.

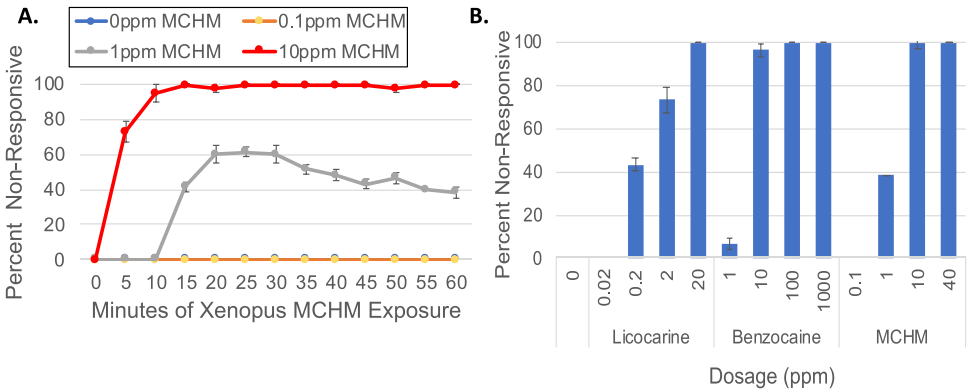

Fig. 2. Non-responsive embryos over a 60-minute incubation with MCHM. A. Stage ~35 embryos were treated with the indicated concentration of MCHM, and responses to gentle touching were recorded as described in *Materials and Methods* (n = 250 per sample, N = 5). **B.** Quantitation of movement with different concentrations of lidocaine benzocaine, and MCHM with concentrations indicated in ppm after one hour of incubation (n = 60 per sample, N = 3).

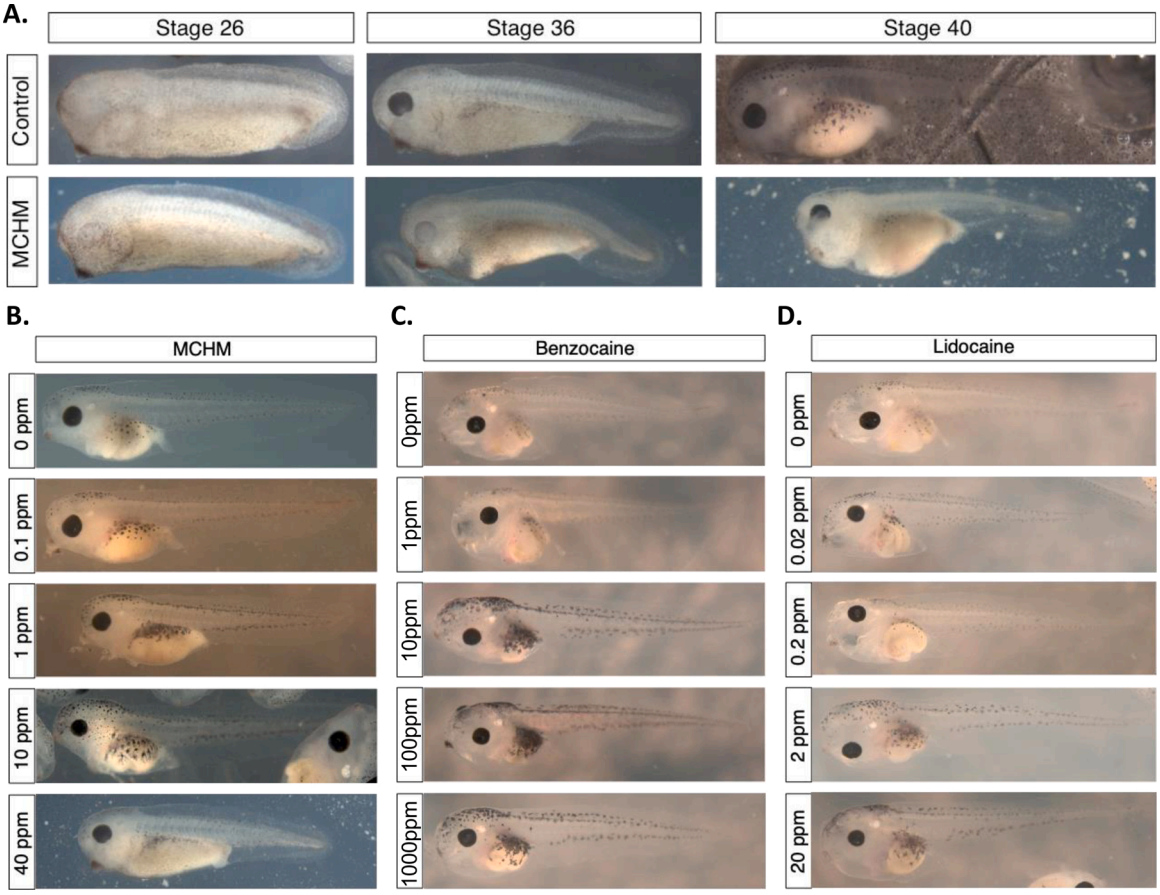

Fig. 3. Developmental defects displayed by surviving embryos with MCHM, benzocaine, and lidocaine. A. *Xenopus* embryo morphology at the indicated stages are shown when cultured in 100 ppm MCHM at NF stage 19 (n = 400 per sample, N = 4). **B.** *Xenopus* embryo morphologies when cultured in different concentrations of MCHM. **C.** *Xenopus* embryo morphologies when cultured in different concentrations of benzocaine. **D.** *Xenopus* embryo morphologies when cultured in different concentrations of lidocaine. For **B-D**, n = 60 per sample, N = 3.

3. Discussion

While the viability of human cell lines exposed to MCHM has been evaluated *in vitro* [9], we further characterized the physiological effects on human cells and *Xenopus tropicalis* embryos which lead to unexpected results. From studies in *Saccharomyces cerevisiae,* ROS levels are implicated in DNA damage [15]. The ability of glutathione treatment to serve as a rescue for MCHM toxicity in HEK293T human cell line suggested that ROS serves a vital role in MCHM toxicity at a cellular level. The effects of glutathione in the response to MCHM could be due to any or several of its homeostatic roles. One possible explanation is that the antioxidant nature of the peptide was required to save the cell from ROS produced by MCHM exposure. Another explanation could be that MCHM interferes with glutathione's activities related to metal ion homeostasis, resulting in cell toxicity due to the requirement of GSH for cell function not related to antioxidant activity [18]. While the damage caused by MCHM-induced ROS occurs in cells, it is not yet possible to conclude to what extent the damage can be conferred at spill levels to

multicellular organisms with robust ROS buffering mechanisms. Our results agree with other reports that MCHM at the highest levels of exposure decreases cellular viability possibly through membrane degradation [9]. It is similarly unclear to what extent treatment with an antioxidant such as glutathione can be conferred to such organisms to mitigate damage.

Our experiments on *Xenopus* embryos revealed a phenotype of MCHM treatment involving apparent non-responsiveness. The non-responsiveness was dose-dependent and immediate at higher doses of exposure to the chemical, and they were reversible after removal of exposure. The potential causes of this phenotype are still under investigation. Non-responsiveness in vertebrates can occur in a myriad of ways, including blocking ion channels, such as voltage-gated sodium channels, potassium channels, or calcium channels or affecting neurotransmitter release or reuptake [32]. Interestingly, valproate, a known antiepileptic drug that can also reduce cellular inositol, increases the amount of time before levamisole induced non-responsiveness in *Caenorhabditis elegans* [33]. Valproate was also implicated to reduce the release of the neurotransmitter acetylcholine, implying a potential connection to inositol and neurotransmitter release [33]. Due to the immediate non-responsive effects of MCHM, a likely cause for MCHM non-responsiveness in the tadpoles is an ion channel or acetylcholine inhibition mechanism and not a mechanism that occurs on a transcriptional timescale, such as the other cellular effects revealed in this study. It is clear that this phenotype must be further explored as it pertains to the impact of chemical spills on wildlife. Our data also showed that MCHM impacts the development of *Xenopus* embryos including the defects in anterior-posterior body axis elongation and reduced eye and body pigment [34]. The latter may be caused by compromised melanization, a developmental process that occurs in both the neural crest-derived melanophores and the neuroepithelium-derived retinal pigment epithelium (RPE) cells. In *Xenopus* and other vertebrates, melanization in both the melanophores/melanocytes and the RPE cells is controlled by the same set of genes, such as those encoding the MITF transcription factor and certain downstream targets [34,35]. The mechanism of these MCHM-induced developmental defects is still under investigation. A previous study on adverse birth outcomes caused by MCHM revealed no correlation of outcomes, such as birth weight, to the location of maternal residence, specifically living within the exposure zone *versus* outside [6]. This result is hopefully indicative of minimal effects on the local population. However, given our cellular and embryological data, long term studies of MCHM on animals, including humans, may uncover more subtle effects on development and health.

Our study of MCHM highlights a general lack of knowledge about many of the chemicals that surround us in our daily lives. This chemical has been in use for the processing of coal for decades with little awareness of its potential health consequences should it leave controlled storage [36]. The storage of this chemical was ripe for accidentally contaminating drinking water based on its location [5]. Assuming uncharacterized chemicals are safe enough for such storage is unlikely to be the most prudent policy. The MCHM spill and the studies that followed point to a need to at least treat these chemicals with increased care based on their potential as toxins. For the safety of the public, they should be treated as possible sources of a health crisis so that at least their storage conditions cannot lead to one.

4. Materials and methods

4.1. Xenopus embryo culturing and manipulation

Xenopus tropicalis embryos were collected and cultured as described previously [30]. For studies of embryonic development, embryos were cultured post-fertilization in 0.1X Modified Barth's Saline (MBS) supplemented with 0.28 mM MCHM at room temperature (~21 °C) until stage ~40, and gastrointestinal tracts, melanocyte size, and heart activity were monitored at various stages. For the non-responsive phenotype, embryos were cultured in 0.1X MBS to stage ~35 before being treated with various concentrations of MCHM, lidocaine (MP Biomedicals 6108-17-4), and benzocaine (Sigma 94,097) and the 60-minute time course was performed with 10 embryos per treatment. The embryos were gently touched every 5 min with a hair loop and then observed for movement. Viability in the Xenopus embryos was determined by the embryos' turning white with the tissue sloughing off and becoming soft. It was further determined by the embryos swelling at stage 19 and shrinking at stage 26 and 38.

4.2. MTT assay

96-well plates were seeded with 5×10^3 HEK293 T cells per well. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) at 37°C with 5% CO_2. 24 h after seeding, the medium was replaced with L15 medium supplemented with 10 % FBS and MCHM at the indicated concentration and indicated chemicals (1 mM or 10 mM glutathione, 100 u M vitamin C, 0.5 mM EGTA, 25 mM $CaCl_2$, 5 u M A23187, and 2ug/mL tunicamycin), and cells were cultured at 37°C without CO_2 supply in a separate room. MCHM is a volatile chemical and causes headaches and nausea [37]. L15 media is designed for cultures without CO_2 supply. After 24 -h culturing, 10μL of 5 mg/mL of MTT was added to each well (final concentration 0.5 mg/mL), and the plates were incubated in the dark for 4 h. The cells were then lysed, and the formazan crystals were solubilized by adding 100 μl of acidic 10 % SDS to each well. The plates were then incubated for another 4 h, and absorbance was measured at 570 nm.

4.3. Cell counting

10 cm culture dishes were seeded with 5×10^6 cells and grown in DMEM supplemented with 10 % FBS for 48 h. The media was then changed with DMEM supplemented with FBS and indicated treatments. At 72 h the cells were resuspended and counted on a hemocytometer.

4.4. Qualifying the pigmentation of the embryos

Embryo melanocytes were qualified by the amount of coverage (black area) there was over the developing gastrointestinal tract (yellow-white area). They were qualified as either high or normal pigment.

Author statement

MP and SK carried out *Xenopus* and mammalian cell lines. MCA co-designed the study and co-wrote the paper with MP. SW supervised MP and SK. JEGG designed the study, supervised the project, and co-wrote the paper.

Author contributions

MP and SK carried out *Xenopus* and mammalian cell lines. MCA co-designed the study and co-wrote the paper. SW supervised MP and SK. JEGG designed the study, supervised the project, and co-wrote the paper.

Ethical approval and informed consent

Methods involving live animals were carried out in accordance with the guidelines and regulations approved and enforced by the Institutional Animal Care and Use Committee at West Virginia University.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.12.009.

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