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Head and Heart 2022 Research Output

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ABSTRACT

Indigenous communities in Canada often struggle with access to clean drinking water. Chlorination is the primary water disinfection method in these communities, but due to high levels of organic carbon in the water pre-disinfection, the likelihood of chloroform formation, a trihalomethane (THM), is significantly increased. In 2019, Eabametoong and Attawapiskat First Nations in Northern Ontario declared a state of emergency due to high levels of THMs, mainly chloroform, in their drinking water. Health Canada supports that the benefits of disinfection through chlorination outweigh the risks of long-term, low-dose chloroform exposure. However, the field is lacking in research on the long-term effects on complex human health outcomes. This study aimed to assess the effects of low-dose chloroform treatment on a biomarker of health, mitochondrial DNA copy number (mtDNA-CN). mtDNA-CN variation is known to be associated with aging, frailty, and mortality. After 48h of low-dose chloroform treatment in HEK293 cells, no dose dependent mtDNA-CN changes were seen. HEK293 cells were then treated with a higher range of chloroform doses for 48h and yielded the same observation of no significant mtDNA-CN variation. Although there were no significant observations, this experiment tested only one biomarker of health in one cell line and was not conducted in a long-term setting, thus conclusions about the long-term health effects of low-dose chloroform exposure require further investigation.

POSITIONALITY STATEMENT

Before you read about my Head and Heart project, I'd like to tell you about myself. My name is Amanda Louise Morin. I was named after my paternal aunt (Amanda) and my maternal grandma (Louise) and bear the last name of my maternal grandfather. I am Ojibwe and European. My dad's family is from Wikwemikong Unceded First Nation on Manitoulin Island, and my mom's family is French Canadian and British. It is important to note that while I strongly identify with both of my ethnicities, I was raised by my mom in a small Amish and Christian town. I usually phrase this as being "raised white", as I grew up with virtually no connection to Indigenous culture. I did excel academically, and I have been fascinated with science basically my whole life. I knew at 16 that I wanted to study biology here at Western. After 21 years of being alive, I had finally connected with the Indigenous Student Center at Western in my third year. Since then, it has been quite the journey learning how to walk in two worlds. I've also started to realize that I've been walking these two worlds since the moment I was born, even though I was "raised white". I have many examples, but I'm going to give you a more personal example.

I've struggled with my mental health for basically my entire life. I was a shy and anxious kid who was always deemed too sensitive and too distracted. I was a straight-A student and didn't get into a lot of trouble, so no one ever really thought too much about my always being quiet and reserved. This barrelled into anxiety and depression at a really young age (12 years old). Looking back, I always pondered why I was the way that I was. I believe it was because my spirit was straddling two worlds, but my physical body was in an environment that didn't foster any Indigeneity. Until my third year at Western when I began my quest of connecting with my culture, I really felt as if the body I was in wasn't supposed to exist. Things never felt completely "right".

My Indigeneity saved my life. I am here today because of my love for and connection with the Earth. I've finally made my connection to my culture, and I feel quite strongly that I can live my absolute best life by bringing my Indigeneity into everything that I do. That also includes my research.

I believe in blood memories. I believe that intergenerational trauma exists, and I believe there is a way to observe this phenomenon. Our DNA comes from hundreds of generations before us, and I believe DNA holds the story of our existence. I believe blood memories can be unraveled by studying genetics, and understanding how shared trauma over the course of centuries has shaped DNA and gene expression. It is important for me that my research pertains to obtaining knowledge about DNA, as any new information will help unravel the story of blood memories.

For my Head and Heart project, I felt strongly that the research should pertain to an issue that is experienced by Indigenous populations. In previous fellowships, my research pertained to the health of the water. Because Indigenous people believe water is a living entity, and because many Indigenous communities experience a lack of access to clean drinking water, I decided that my project would look at the effects of water contaminants on mitochondrial DNA, thus aligning my research beliefs with the tools available to me in the lab.

I hope you are able to read my paper with the understanding that I am writing from both an academic and a personal perspective. I like to make my research personal, and I believe I have done that well here. I hope you are able to sense my passion for both the sciences and my culture upon reading this report. Miigwetch (thank you) for taking the time to read my report.

INTRODUCTION

Chloroform is a trihalomethane (THM) with the chemical structure $CHCl_3$ and is the most abundant THM disinfection by-product (DPB) present in potable water[1]. Most chloroform in potable water is formed during chlorination, an efficient and cost-effective water disinfection process. During this process, a series of chemical reactions between chlorine and organic carbon present in the water occur, ultimately forming chloroform[2]. When more organic carbon is present in the water to be disinfected, the amount of chloroform by-product increases.

Ontario's lakes, including the Great Lakes, hold about 20% of the entire world's fresh water[3]. In Northern Ontario, several remote Indigenous communities are scattered among the many lakes. Drinking water for these remote communities is derived from the surrounding lakes and rivers and is disinfected via chlorination[4]. Eabamet Lake acts as the water source for Eabametoong First Nation, a remote fly-in Indigenous community ~300km north of Thunder Bay. In 2012 and 2013, this lake contained 5.5x the amount of dissolved organic carbon than the Great Lakes combined[5,6]. Additionally, the primary method of water disinfection in remote Indigenous communities is chlorination[4]. As such, the water of many remote Indigenous communities has levels of chloroform higher than the maximum acceptable concentration (MAC) of all THMs, a metric determined by both the provincial and federal governments (0.1ug/mL)[7,8]. In fact, Eabametoong First Nation declared a state of emergency in July 2019 when the communities potable water was revealed to contain 0.25ug/mL of chloroform, 150% higher than the MAC for all THMs[9]. Eabametoong First Nation has been under a boil-water advisory since 2001[10]. However, a state of emergency declaration was necessary in the case of THM contamination because a one-minute boil does not effectively remove chloroform or other THMs from water[11].

Health Canada supports that the benefits of water disinfection through chlorination far outweigh the long-term low-dose exposure to chloroform and other THMs in potable water[12], however adverse health effects associated with long-term, low-dose chloroform exposure are not well known or understood. Chloroform was once commonly used at high doses as an anaesthetic

for surgery and other painful medical procedures, but stopped being used in these clinical settings in 1976[13] so the necessity of studying its effects on human health has deflated significantly. Current studies of the effects of chloroform *in vitro* and *in vivo* use high doses of chloroform for no more than 96 hours[14,15,16,17,18]. This study was inspired by the reality that many remote Indigenous communities do not have reliable access to clean drinking water, and are continuously exposed to potentially harmful DBPs such as the THM chloroform. As such, this study hypothesized that chloroform exposure may affect a key biomarker of health well known to be modified by chemical exposures, mitochondrial DNA copy number (mtDNA-CN)[19].

The mitochondrion is often referred to as the powerhouse of the cell, since the majority of chemical energy required by a cell is produced in this organelle. The mitochondrion contains its own genome separate from nuclear DNA, called mitochondrial DNA (mtDNA). mtDNA encodes 13 oxidative phosphorylation (OXPHOS) proteins (OXPHOS is how a cell generates the majority of its energy), as well as 22 tRNAs and 2 rRNAs that are required for translation. One cell has multiple mitochondria, and each mitochondrion can hold hundreds of copies of mtDNA. A cell with more copies of mtDNA have a higher mtDNA-CN, whereas the opposite is true for lower mtDNA-CN. Different cell types have different baseline mtDNA-CN levels to account for the varying energy demands of the body's multitude of tissues. For example, lung epithelial cells (a component of lung tissue) have significantly lower mtDNA-CN than cardiomyocytes (a component of heart tissue)[20] because lung tissue doesn't need as much energy to function as heart tissue does. When mtDNA-CN strays from its baseline level, the supplementation of energy no longer matches the demands of the cell, and this can have adverse effects on the health of the cell and may contribute to cell death[21].

mtDNA-CN variation plays an important role in health and disease. Females generally have a higher baseline mtDNA-CN in all tissues compared to males[22]. mtDNA-CN decreases with age[23], and is also associated with frailty and all-cause mortality[22]. Low mtDNA-CN may contribute to cardiovascular disease[24]. mtDNA-CN variation has been hypothesized to be a key factor in the

pathologies of other complex diseases such as autism, Alzheimer's, Parkinson's and Huntington's to name a few[25,26].

We are interested in the role mtDNA variation and dynamics play in the pathology of complex diseases. As such, we have optimized the estimation of mtDNA-CN in a cost-effective manner using qPCR. This allowed us to assess the effects of chloroform exposure on mtDNA-CN in a human kidney cell line. It was hypothesized that low-dose chloroform exposure would alter mtDNA-CN and thereby have potential implications for human health and disease.

METHODS

Cell Culture

HEK293 cells (ATCC, CRL-1573) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Pen-Strep at 5% CO_2 and 37^{o}C. To harvest, media was removed and cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Thermo-Fisher). Cells were incubated in 0.05% Trypsin-EDTA solution to detach them from the plate, and the Trypsin-EDTA solution is neutralized with supplemented DMEM. All cell culture reagents were obtained from Thermo-Fisher.

Chloroform Treatment

Two chloroform treatment experiments were conducted. HEK293 cells were harvested as described above at 90% confluency. Cell density and viability were determined via Trypan Blue exclusion in the Countess II (Invitrogen) and diluted to 100 000 live cells/mL. Cells were plated in a 6-well dish at 200 000 cells/well. Cells were allowed 24h to attach to the plate and were then treated in triplicate with increasing doses of chloroform dissolved in 100% ethanol. For the first experiment, doses were 0.02, 0.07, 0.1, and 0.25ug/mL. Doses were selected as biologically relevant doses as determined by THM/chloroform levels in drinking water from several North American locations as well as government regulations for MAC of THMs (Table 1). For the second

experiment, doses were 0.25, 115, 230, 345 and 460ug/mL. The high dose of 460ug/mL was determined from the literature as a dose of chloroform that results in >80% cell viability after 48 hours of treatment[15]. The low dose of this experiment is the high dose from the last experiment. Incremental doses were then calculated by dividing 460ug/mL by 4 to give 5 doses and a vehicle control to treat HEK193 cells in triplicate in 3 6-well plates. After 48h chemical exposure, cells were harvested for phenol:chloroform:isoamyl alcohol DNA extraction and ethanol precipitation. DNA was subsequently cleaned twice by ethanol precipitation to improve 260/230 values.

Table 1. Chloroform doses found in disinfected (chlorinated) water from different locations, or from government threshold.

Chloroform dose (ug/mL)	Location/Source
0.02	London, ON, Canada
0.07	Cobb County, Georgia, USA
0.1	Health Canada MAC for THMs
0.25	Eabametoong First Nation

mtDNA-CN estimation via qPCR

Mitochondrial DNA content is determined by calculating the difference between the Ct value for the mitochondrial probe and the Ct value for the nuclear probe. Because the nuclear genome is present in one copy and the mitochondrial genome is present in multiple copies, this provides a ratio of the multiple copies of the mitochondrial genome normalized to the single copy of the nuclear genome. mtDNA-CN was determined via single-plex real-time quantitative polymerase chain reaction (qPCR) on Applied Biosystems Step One Real-Time PCR System using SYBR reagents and reverse and forward primers for Albumin (conserved nuclear gene, one copy) and D-loop (control region of mtDNA, multiple copies). The thermal cycling program started with enzyme activation at 95^{o}C for 15 minutes, followed by 45 cycles of 94^{o}C for 15s, 62^{o}C for 10s, and 74^{o}C for 19s with data collected at 74^{o}C.

RESULTS

HEK293 cells were treated with biologically relevant doses of chloroform (0.02–0.25ug/mL, Table 1) for 48 hours following which DNA was extracted and cleaned. qPCR analysis revealed that mtDNA-CN increased significantly when cells were treated with 0.02 and 0.07 ug/mL of chloroform as indicated by a mean delta C_t increase of 0.63 and 0.97 compared to vehicle, respectively. mtDNA-CN also significantly increased in cells treated with 0.1 and 0.25 ug/mL of chloroform compared to the vehicle control, but significantly decreased compared to the mtDNA-CN of cells treated with 0.07ug/mL of chloroform (Figure 1). Given the small increases in mtDNA-CN between low doses, we next wanted to assess if there are dose-dependent effects on mtDNA-CN in cells treated with higher doses of chloroform.

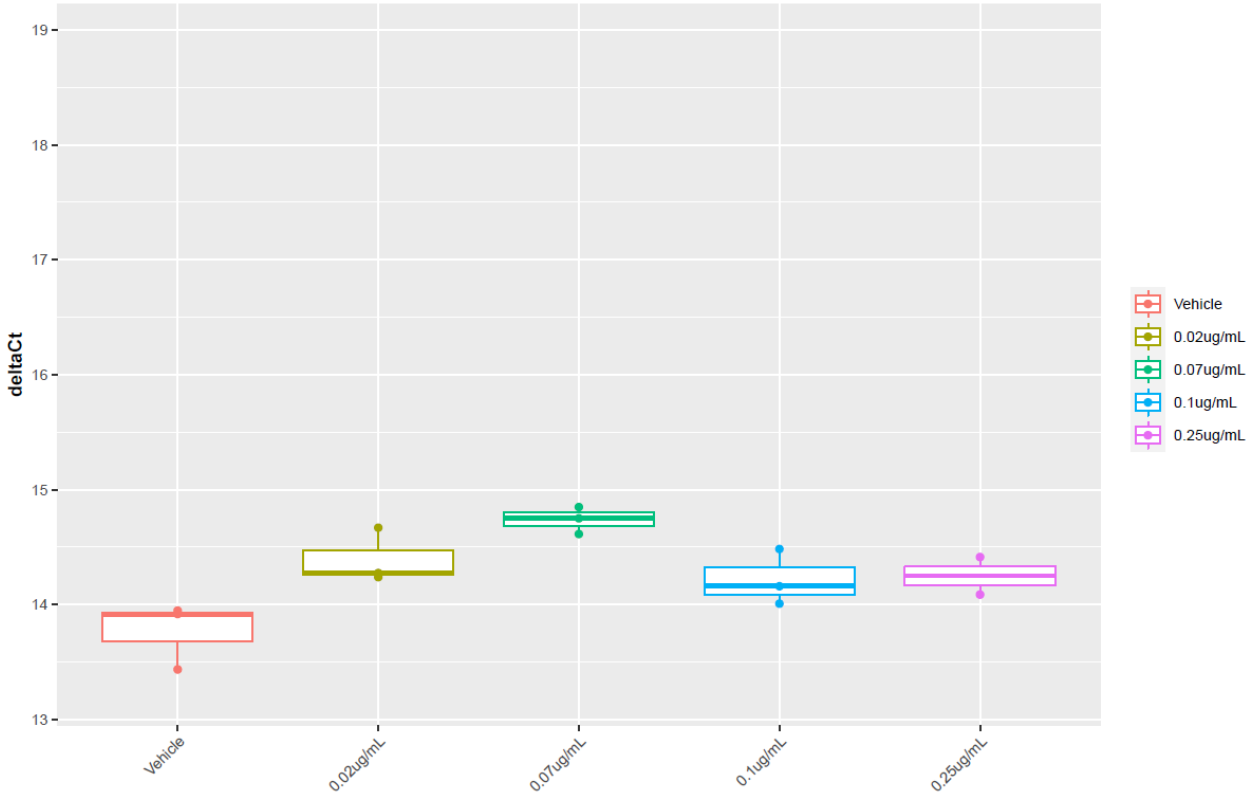

Figure 1. mtDNA-CN estimates from delta Ct values determined by qPCR in HEK293 cells treated with 0.02, 0.07, 0.1 and 0.25 ug/mL chloroform. A higher delta Ct value indicates an increase in mtDNA-CN.

HEK293 cells were then treated with increasing doses of chloroform ranging from 0.25–460ug/mL for 48h followed by DNA extraction and cleanup. qPCR analysis showed significant increases in mtDNA-CN in all doses compared to vehicle control (Figure 2). Each dose had a significantly higher mtDNA-CN than the previous dose, with the exception of 460ug/mL treated cells, whose mtDNA-CN does not significantly differ from that of 230ug/mL treated cells.

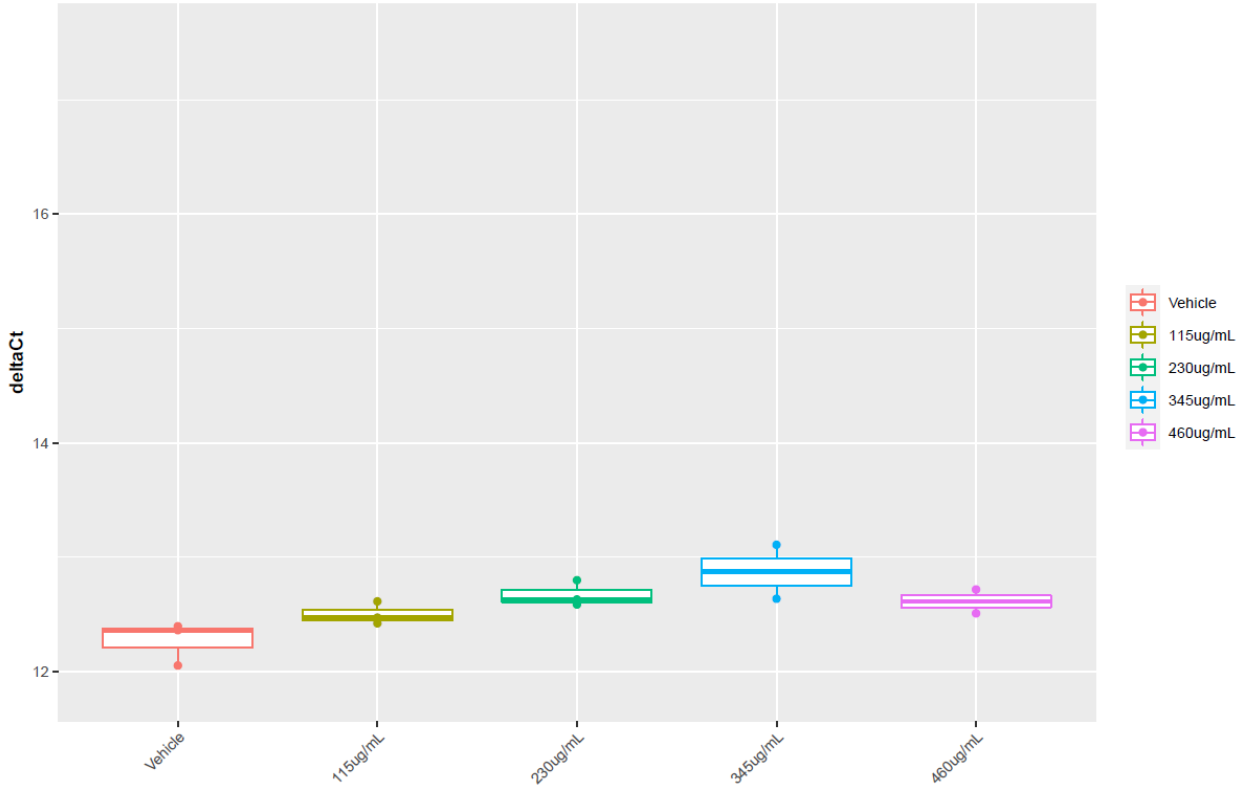

Figure 2. mtDNA-CN estimates from delta Ct values determined by qPCR in HEK293 cells treated with 115, 230, 345 and 460 ug/mL chloroform. A higher delta Ct value indicates an increase in mtDNA-CN.

DISCUSSION

Chloroform is the most abundant THM present in potable water as a DBP, and it's content is highly correlated with levels of organic carbon present in the water pre-disinfection[1]. Lakes and rivers in Northern Ontario, water sources for remote Northern Indigenous communities, have significantly higher levels of organic carbon compared to the Great Lakes, the main water source

for Southwestern Ontario[5,6]. Furthermore, chlorination is the disinfection method of choice for remote Northern Indigenous communities, due to cost-effectiveness and efficiency[4]. Unfortunately, this means that remote Northern Indigenous communities are susceptible to experiencing higher than the provincially and federally determined MAC of THMs in their potable water. In fact, in July 2019, two remote Northern Indigenous communities, Attawapiskat and Eabamatoong Nations, declared states of emergency when THM levels were detected to be over 100% higher than Health Canada's MAC for THMs[9,28]. Chloroform is not effectively removed from water after one minute of boiling[11], so a boil-water advisory in response to high THM levels is not feasible. Although long-term low-dose exposure to chloroform is thought to be harmful to human health, few studies have shown this. As such, Health Canada supports that the benefits of water decontamination through chlorination outweigh the potential risks associated with the consumption of chloroform and other THM DBPs[12].

The results indicate that cells treated with chloroform experience a significant increase in mtDNA-CN compared to the vehicle control (Figures 1 and 2). It is known that increased mtDNA-CN is positively associated with mitochondrial function[27]. In this experiment, increased mtDNA-CN may be a compensatory response to chemical insult. Alternatively, the variation of mtDNA-CN seen may be due to natural variance of steady-state mtDNA-CN in HEK293 cells. We conclude that chloroform is able to invoke subtle changes in mtDNA-CN at certain concentrations but this does not appear to occur in a dose dependent manner. With the knowledge that low doses of chloroform may evoke a small mitochondrial response, the next question pondered was if mtDNA-CN varied in a dose-dependent manner upon exposure to higher doses of chloroform. The literature was assessed to determine a high dose of chloroform (see Methods). Based on this, the doses of chloroform decided upon were 0.25, 115, 230, 345 and 460 ug/mL. Results from this experiment show a pattern of mtDNA-CN increase similar to that of the first experiment, wherein each dose of chloroform evoked a significant increase in mtDNA-CN compared to vehicle control, but the increase in mtDNA-CN was not dose-dependent. Observing the same pattern of

mtDNA-CN variation in two experiments using significantly different doses of chloroform points to the possibility that chloroform exposure does not have a dose dependent effect on mtDNA-CN and may suggest that the cells can perform some sort of compensatory mechanism in response to a chemical insult.

Presently, there are no studies connecting chloroform treatment specifically with mtDNA variation. One study observed reduced mitochondrial membrane potential and metabolism after exposure to 835 and 1050 ug/mL of chloroform[14]. Another study saw significant oxidative stress associated with exposure to doses of chloroform 955ug/mL and higher[15]. That said, these doses of chloroform far exceed the doses anyone would experience by consuming or bathing in contaminated potable water. Furthermore, these studies were performed *in vitro* for less than 72 hours.

There are few studies that look at the effect of chloroform exposure on different human cell types. One study collected whole blood samples from healthy human subjects that were exposed to 10, 30 and 50 ug/mL of chloroform and found that hematocrit and white blood cell count were only mildly affected[16]. Another study collected lung epithelial cells from healthy human subjects that were exposed to 1.19, 11.9 and 119 ug/mL of chloroform, which resulted in non-significant DNA damage[17]. Finally, in a study using human hepatocyte cell line HepG2, cells were treated with 119, 477, 955 and 2388 ug/mL chloroform which resulted in single stranded DNA breaks and significant lipid peroxidation from oxidative stress[15]. Although these studies were short-term and used doses of chloroform higher than the doses anyone would experience by consuming or bathing in contaminated potable water, the observation of DNA damage, oxidative stress and perturbed complete blood count in human cells provides indirect evidence that low dose chloroform exposure is likely to exert adverse health effects over time.

Communities with high THM and chloroform levels in their potable water are likely to experience adverse health effects from long-term, low-dose exposure, yet there are no studies observing the long-term effects of low-dose chloroform exposure *in vivo* in cohorts or animal

models. There was a small cohort study conducted in the U.S. states of Texas and Georgia where women of reproductive age had their blood taken and assessed for chloroform levels before and after showering for 30 minutes in water from at least two separate locations[18]. In Texas, median chloroform blood levels for the cohort increased from 25ppt to 57ppt after a 30-minute shower in water tested to contain 0.008ug/mL of chloroform. In Georgia, median chloroform blood levels for the cohort increased from 70ppt to 280ppt after a 30-minute shower in water tested to contain 0.085ug/mL of chloroform. The concentration of chloroform in the water in these two states falls underneath Health Canada's MAC of THMs in drinking water (0.1ug/mL), yet blood chloroform levels increased significantly after a single 30-minute shower. Although this study only had one data collection point for blood chloroform levels, it begs the argument that blood chloroform levels regularly increase upon exposure to water containing chloroform, and that the likelihood of long-term toxicity due to low-dose chloroform exposure warrants further investigation. It is also pertinent to note that the water in Texas had significantly lower chloroform levels due to water disinfection by chloramination, rather than chlorination. Chloramination is the process of disinfecting water with the addition of chloramines. Chloramines are more stable than chlorine, thus chloramination attenuates the formation of THMs and other DBPs compared to chlorination[29]. This is solid evidence that formation of DBPs in drinking water can be avoided using disinfection systems that are more advanced than chlorination.

It is important that studies of long-term, low-dose chloroform exposure continue. This study looked at a single biomarker of health, mtDNA-CN. Although no dose dependent effects of chloroform on mtDNA-CN were seen, this is not conclusive evidence that chloroform does not negatively affect health. Future research should continue *in vitro* studies that interrogate the effects of chloroform exposure on a multitude of health biomarkers in various cell lines. A future experiment could expose THP1 cells, a monocytic (white blood cell) cell line to chloroform as they are more representative of the matured blood cells that would absorb chloroform from the gastrointestinal tract *in vivo*. Future research could also use an animal model to exhibit the long-

term effects of low-dose chloroform exposure, or a longitudinal cohort study with participants from remote Indigenous communities living with higher than the MAC of THMs in their drinking water.

CONCLUSION

The literature supporting adverse health outcomes associated with chloroform exposure is limited. Results here indicate that chloroform exposure significantly increases mtDNA-CN, a biomarker of health, however not in a dose-dependent manner. We suggest that adverse health outcomes of chloroform exposure should be studied more extensively, assessing multiple biomarkers of health in different cell lines, at a variety of timepoints, and in animal or cohort studies. Any information generated will be beneficial to Indigenous communities with high THM levels in their potable water, and of course will also be beneficial to any other community who is faced with the same obstacles to clean drinking water, and could be the push needed to rationalize more advanced water filtration systems in these communities.

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Finally I would like to acknowledge my supervisor and mentor, Dr. Christina Castellani. She allowed me to create my own research project from start to finish, which allowed me to explore myself as a researcher and as an Indigenous-European woman in a really meaningful way. Thank you so much for this experience!

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