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Micronuclei formation in rainbow trout cells exposed to multiple stressors: Morpholine, heat shock, and ionizing radiation

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ABSTRACT

Discharges from industrial cooling water systems can include low levels of morpholine (a chemical pH regulator and corrosion inhibitor), as well as transiently higher temperature effluent water which present a potential source of environmental impact to aquatic biota. The effects of environmental levels of morpholine or heat shock (HS) treatment alone and in combination with a challenge high-dose of ^{137}Cs ionizing radiation were studied using the cytokinesis block micronucleus assay in a rainbow trout cell line (RTG-2). Morpholine treatment of 10 or 100 mg L^{-1} alone produced no significant effects, and no interaction was observed in combination with 7.75 Gy radiation. A 9 °C magnitude HS treatment alone significantly increased micronuclei formation. A synergistic response was observed when 9 °C HS was combined with 7.75 Gy radiation, with 15% more cells containing 3 or more micronuclei than the sum of each individual stressor. A synergistic increase in the average number of micronuclei was observed when morpholine and a 9 °C HS were co-treated. These results indicate that morpholine at environmentally-relevant levels does not impact micronuclei formation or cell cycle progression however 9 °C HS may be of potential concern both alone and in combination with other stressor treatments.

1. Introduction

Aquatic ecosystems are exposed to a variety of stressors resulting from human activities such as thermal power generation. Many thermal power plants located near large natural bodies of water rely on once-through cooling during the process of electricity generation (Kelso and Milburn, 1979). Source water is used to condense turbine steam and is subsequently discharged back into the environment at an elevated temperature compared to ambient conditions. Aquatic species found near these discharges may be exposed to various stresses, including thermal stress resulting from warm water releases and low levels of chemicals (e.g. morpholine) that are added to cooling water systems to prevent corrosion and biofouling (Eloranta, 1983; Brungs, 1973). It is therefore important to understand the potential impacts that these exposures may have on aquatic biota.

Environmental levels of thermal or chemical stressors are often below lethal levels but may impact species at the cell or tissue level. DNA or chromosomal damage, if unrepaired or repaired incorrectly, can lead to longer-lasting impacts such as genomic instability and multigenerational effects and is therefore an important endpoint to consider when assessing potential risk (Spry et al., 2007). A common tool for quantifying genotoxic damage is the cytokinesis-block micronucleus (CBMN) assay, which measures the formation of micronuclei (MN) consisting of acentric, dicentric, fragmented or whole chromosomes which are improperly segregated during mitosis (Fenech, 2000). Cells in this assay can be quantified for two things; the number of micronuclei and the number of full size nuclei. The number of micronuclei correlates to the amount of genotoxic damage to a cell. The number of full size nuclei gives an indication of cell cycle progression, since the addition of cytochlasin-b blocks cytokinesis but not karyokinesis (Fenech, 2000). For example, the presence of a binucleate cell (with two full size nuclei), indicates the completion of one cell cycle. A

Abbreviations: CBMN, cytokinesis-block micronucleus; Gy, Gray (unit of absorbed dose); HS, heat shock; MN, micronucleus (micronuclei); RTG-2, Rainbow Trout Gonad-2

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decrease in the ratio of binucleate cells to mononucleate cells suggests a delay in cell cycle progression.

In nature, organisms are rarely subjected to stressors individually, but are exposed to multiple stressors simultaneously. Combined stressors can have complex interactions. It may be the case where a single exposure in isolation does not produce a measurable effect for a given endpoint, but may alter the response to a second high-dose exposure (Dale Becker and Wolford, 1980; Stone et al., 2001). The combined cumulative effect (response 'C') may be broadly classified as being additive (C = A + B), synergistic (C > A + B) or adaptive (C < A + B); where A is the response to a single stressor 'X', B is the response to a different stressor 'Y' and C is the response when treated with both X and Y (Crain et al., 2008). Combining low-dose environmentally relevant exposures with a high-dose damage inducing stress can provide further insight into the physiological response to these stressors that may not be observable with a single low-dose treatment alone.

Morpholine (C_4H_9NO; CAS no.: 110-91-8; EC no.: 203-815-1) is a heterocyclic amine and a strong base (pK_b = 5.6; Mjos, 1978). It is used as a corrosion inhibitor, functioning as a neutralizing amine for carbonic acid in steam boiler systems (Bloom, 2003). The United States Food and Drug Administration limits the concentration of morpholine in steam condensate to 10 mg L^{-1} (Kolliopoulos et al., 2015). Despite its industrial use and potential release into the aquatic environment, biological studies examining the effects of morpholine are limited. Studies on aquatic biota have only examined whole organism toxicity across a select number of species, with LD_{50} values ranging from 250 to 500 mg L^{-1} (Dawson et al., 1975; Juhnke and Lüdemann, 1978). Studies on the effects of morpholine at the cellular level are very limited, with a single report of an NI_{50} value (concentration of compound which results in a 50% reduction in neutral red uptake, a measure of cell viability) of 8027 mg L^{-1} for morpholine in cultured fathead minnow fish cells (Brandão et al., 1992). *In-vitro* studies such as cell culture models would allow for investigation into potential mechanisms of toxicity at high doses.

Cooling water discharges, at the point of release, have been reported to be upwards of 10 °C above intake (Madden et al., 2013). While the warmer water will dilute out after release, substrate monitoring at fish spawning grounds near discharges have found that water temperature on the lake bottom can be up to 3 °C above ambient (Griffiths, 1987; Thome et al., 2016). The molecular response to heat shock has been well studied, but there is controversy regarding whether thermal stress can independently induce genotoxic damage (Takahashi, 2004; Kampinga, 2005; Dong et al., 2007; Hintzsche et al., 2012). There is also evidence in a variety of systems that a priming heat shock can induce an adaptive response and reduce genotoxic damage from a follow-up high-dose stress (Cypser and Johnson, 2002; Shen et al., 1991; Boreham et al., 1997; Yellon et al., 1992). These studies illustrate the complexity of assessing risk of thermal stress (particularly when combined with an additional stressor) and the need for further study.

The purpose of this study was to examine the genotoxic effects of exposure to morpholine and acute thermal stress using an *in-vitro* cell culture model. Rainbow trout cells were exposed to thermal heat stress or morpholine individually at environmental levels or slightly above (within one order of magnitude), and chromosome damage was assessed through quantifying micronuclei formation. We hypothesized that morpholine levels significantly above environmental levels (tested concentrations of 100 or 1000 mg L^{-1}) would result in increased micronucleus formation and/or delays in cell cycle progression. As all tested magnitudes of heat shock were within ranges experienced transiently in the environment, we did not expect significant effects of heat shock treatment alone. In addition to examining the induction of damage in isolation, both morpholine and heat shock were coupled with a high-dose ionizing radiation exposure to investigate how a low-dose environmentally-relevant stress can alter the response to a high-dose damage inducing treatment. High doses of ionizing radiation are known to induce micronuclei formation, and we were interested in studying the possible modification of this damage when co-treated with an environmentally-relevant heat shock or morpholine treatment. Due to the hypothesis that environmentally-relevant levels of temperature and morpholine would not induce significant micronuclei formation on their own, by coupling these treatments with a high-dose stressor that causes significant damage, interactions may be observed. Further, the nature of the interaction (synergism or antagonism) could be determined in the same experiment. The presence of interactions, and the nature of that interaction, could suggest possible mechanisms of action, due to the requirement of an overlap in underlying mechanism to observe these interactions. For example, we suspected that due to known overlapping mechanisms, heat shock and ionizing radiation treatment may result in adaptive or synergistic interactions when treated in combination.

2. Materials and methods

2.1. Cell culture

The immortalized rainbow trout (*Oncorhynchus mykiss*) gonadal cell line RTG-2 (ATCC® CCL-55™) was cultured in α-MEM media (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Lonza, Mississauga, Ontario, Canada), 2.5% HEPES buffer (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid; Lonza), 1% penicillin-streptomycin (Lonza), and 1% L-glutamine (Lonza). Cells were cultured at 20 °C under normal atmospheric O_2 and CO_2 conditions.

2.2. Cytokinesis block micronucleus assay (CBMN)

Experiments were completed in 25 cm^2 cell culture flasks initially inoculated with 4×10^5 cells in 5 mL of supplemented media. Flasks were incubated for 48 h prior to initiation of the CBMN assay, during which time they were treated with morpholine, HS and/or radiation as described below. Treatments were applied while cells were in the exponential growth phase. Following treatment, cells were incubated in media containing 3 μg mL^{-1} of cytochlasin B (Sigma-Aldrich, Oakville, Ontario, Canada) for 48 h at 20 °C prior to fixing. The initial density and the length of time cells were exposed to cytochlasin B was optimized for this cell line to provide the highest baseline ratio of binucleate cells for scoring purposes (Supplementary Fig. S1).

Cells were fixed in the culture flasks. To fix, cells were first washed with PBS then given a hypotonic treatment of 5 mL of 0.056 M KCl for 5 min. Cells were soft fixed by adding 5 mL of 3:1 fix (methanol:glacial acetic) to the KCl for 10 min. KCl and fix were removed and 5 mL of fresh 3:1 fix was added for 15 min. Following fixing, cells were air dried overnight.

Cells were stained with 10 mg L^{-1} acridine orange (Sigma-Aldrich) for 30 s then washed in distilled water for 1 min, following which a cover slip was applied to the slide. Micronuclei were identified and scored according to the protocol by Fenech (2000). Scoring was done using a Zeiss Axioplan2 Imaging microscope (Carl Zeiss AG, Oberkochen, Germany; Ex. = 470 ± 40 nm, Em. = 525 nm). The number of micronuclei per cell was scored across 1000 binucleate cells. Cells with three or more micronuclei were scored as 3 + MN, due to the inability of accurately distinguishing individual micronuclei within cells when large numbers were present. When calculating the average number of micronuclei per cell, a conservative estimate was made by rounding down and assuming that all cells with 3 + MN had only 3 MN, in order to increase accuracy of scoring across the study. In addition, the mono-, bi-, tri- and tetra-nucleated frequency (frequency of cells with one, two, three or four complete nuclei respectively) was scored across 1000 randomly selected cells. Individuals scoring flasks were blinded to the treatment dose/concentration.