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Cellular adverse actions of dibromoacetonitrile, a by-product in water bacterial control, at sublethal levels in rat thymocytes

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ABSTRACT

The aim of this study was to investigate the effects of dibromoacetonitrile (DBAN), a by-product in water bacterial control, at sublethal concentrations on rat thymocytes, by using a cytometric technique with appropriate fluorescent dyes. By using this method, the possibility that DBAN induces cellular actions related to oxidative stress was assessed. DBAN reduced the content of cellular nonprotein thiols under Zn^{2+}-free conditions. It elevated the intracellular level of Zn^{2+}, being independent from external Zn^{2+}. DBAN increased cell vulnerability to the cytotoxic action of hydrogen peroxide. These actions of DBAN were likely related to oxidative stress. DBAN is formed by the reaction of bromides and chlorinated oxidants during water disinfection. Hydrolysis of 2,2-dibromo-3-nitrilopropionamide, an antimicrobial used in hydraulic fracturing fluids for production of shale gas and oil, produces DBAN. Therefore, the concern regarding the levels of DBAN in industrial water systems is necessary to avoid the environmental risk to humans and wild mammals.

1. Introduction

With regard to drinking water, the disinfection process is absolutely necessary to avoid waterborne infectious diseases. However, some by-products are generated in the disinfection process (Akin et al., 1982). Some haloacetonitrile by-products have been demonstrated to be hazardous, carcinogenic, mutagenic, genotoxic, and teratogenic under respective experimental conditions (for a review, Yu et al., 2014). The four major species of haloacetonitriles in drinking water are dibromoacetonitrile (DBAN), bromochloroacetonitrile, dichloroacetonitrile, and trichloroacetonitrile (Díaz et al., 2008; Plewa and Wagner, 2008). A previous comparative study using Chinese hamster ovary cells revealed that the toxicities of haloacetonitriles were ordered as follows: DBAN > bromoacetonitrile (BAN) > bromochloroacetonitrile > dichloroacetonitrile > trichloroacetonitrile for cytotoxicity, and BAN = DBAN > bromochloroacetonitrile > trichloroacetonitrile > dichloroacetonitrile for genotoxicity (Muellner et al., 2007). Thus, the bromoacetonitriles (BAN and DBAN) were more toxic than the chloroacetonitriles (dichloroacetonitrile and trichloroacetonitrile). BAN depleted levels of cellular thiols including glutathione (GSH), and induced toxicity (Pals et al., 2011). The in vivo applications of DBAN and BAN reduced both superoxide dismutase and glutathione peroxidase activities and increased the level of malonaldehyde in mice (Deng et al., 2017). These results indicated that DBAN and BAN induced oxidative stress. DBAN is also generated by hydrolysis of 2,2-dibromo-3-nitrilopropionamide (DBNPA), an antimicrobial used in hydraulic fracturing fluids (Dow Chemical Company and Dow Answer Center, 2017). It may be more critical because DBNPA, one of major biocides in fracturing fluids, is used in the production of shale gas and oil (Kahrilas et al., 2014; Ferrer and Thurman, 2015). The control of bacteria by antimicrobials including DBAN is necessary to prevent formation of biofilm that disturbs extraction of shale gas and oil.

It was reported that oxidative stress increased intracellular Zn^{2+} concentrations ($[Zn^{2+}]i$) (Slepchenko et al., 2017). Although an excessive increase in $[Zn^{2+}]i$ caused strong oxidative stress (Kanemoto-Kataoka et al., 2017), the cellular content of GSH was increased by the elevation of $[Zn^{2+}]i$ via intracellular Zn^{2+} release elicited by oxidative or chemical stress (Kinazaki et al., 2011; Fukunaga et al., 2014). Zn^{2+} is an intracellular signal (Hirano et al., 2008). It is a possibility that DBAN and BAN increase oxidative stress, which disturbs intracellular Zn^{2+} homeostasis, resulting in adverse (or lethal) actions. We have tested this possibility by the use of cytometric techniques with fluorescent dyes. This study gives some insights into the diverse actions of bromoacetonitriles.

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Table 1
Specific reagents used in this study.

A. Fluorescent probes [manufacturer]	Emission wavelength
Propidium iodide (PI) [molecular probes, Inc., Eugene, OR, USA]	PI: 600 ± 20 nm
FluoZin-3-AM [molecular probes]	FluoZin-3: 530 ± 20 nm
5-Chloromethylfluorescein diacetate (5-CMF-DA) [molecular probes]	5-CMF: 530 ± 20 nm
B. Zinc chelator [Manufacturer]	**Purpose**
Diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid (DTPA) [Dojin chemical laboratory, Kumamoto, Japan]	Chelating external Zn^{2+}
N,N,N′,N′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) [Dojin chemical laboratory]	Chelating intracellular Zn^{2+}

(*) Excitation wavelength was 488 nm for all fluorescent probes.

2. Materials and methods

2.1. Chemicals

DBAN (95% purity) and BAN (99% purity) were obtained from Alfa Aesar (Lancashire, UK) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. Fluorescent probes and chelators of Zn^{2+} are listed in Table 1. Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless mentioned otherwise.

2.2. Animals and cell preparation

The study was approved by Tokushima University, Tokushima, Japan (T29–52). Cell suspension was prepared as described in previous studies (Matsui et al., 2010). Briefly, the thymus glands were excised from Wistar rats (8- to 10-week-old male, Charles River Japan, Shizuoka, Japan) anesthetized with thiopental. Number of animals was 18. The glands were sliced with a razor (FA-10, Feather Safety Razor Co. Ltd., Osaka, Japan) under ice-chilled conditions. Sliced glands were gently dispersed in ice-chilled Tyrode's solution. The solution containing dissociated thymocytes was passed through a mesh (56 μm diameter) to remove tissue residue. The cells were incubated at 36–37 °C for 50–60 min to recover membrane potentials before the experiments. Importantly, the suspension contained small amount of zinc that remained during the preparation (Sakanashi et al., 2009).

Various concentrations of DBAN and BAN (3–100 mM initially dissolved in dimethyl sulfoxide, DMSO) were added to the suspension (1.998 mL per test tube) to achieve final concentrations of 3–100 μM. The cells were treated with DBAN or BAN for 1–3 h at 36–37 °C. The incubation time varied with experimental purpose. A total volume of 100 μL cell suspension was sampled to assess cellular parameters. Acquisition of data from 2×10^3 or 2.5×10^3 cells needed 10–15 s. DMSO at 0.3% or less did not change cell viability of rat thymocytes. No fluorescence was recorded from the reagents except for fluorescent probes.

2.3. Cellular parameter measurements

We recorded the fluorescence of cells treated with fluorescent probes with a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) (Chikahisa et al., 1996). The fluorescence was examined using equipped software (Version 3.06 for CytoACE-150; JASCO, Tokyo, Japan). To assess the change in cell lethality, propidium iodide (PI) was added to the suspensions to achieve a final concentration of 5 μM. PI fluorescence was recorded for 2 min or longer after the dye application. The excitation for PI was 488 nm. The emission was recorded at 600 ± 20 nm. The cells exhibiting PI fluorescence were considered to be dead cells or membrane-comprised cells. 5-CMF-DA was employed to estimate the content of cellular nonprotein thiols, mainly glutathione (Chikahisa et al., 1996). The cells were treated with 500 nM 5-CMF-DA for 30 min prior to the fluorescence measurement. The fluorescence of 5-CMF was recorded from living cells without PI fluorescence. Increase in 5-CMF fluorescence signifies an increase in intracellular thiol content and vice versa. FluoZin-3-AM was applied to monitor the change in $[Zn^{2+}]i$ (Gee et al., 2002). The cells were then incubated with 500 nM FluoZin-3-AM for 60 min before any fluorescence measurements. FluoZin-3 fluorescence was also recorded from living cells without PI fluorescence. Augmentation of FluoZin-3 fluorescence indicates an elevation of $[Zn^{2+}]i$ and vice versa. The excitation wavelength for 5-CMF and FluoZin-3 was 488 nm while their emission was recorded at 530 ± 20 nm.

2.4. Statistical analysis and presentation

Statistical examination was carried out with Tukey's multivariate analysis. P-values < 0.05 were considered statistically significant. Each experimental series was performed thrice, unless stated otherwise.

3. Results

3.1. Increase in cell lethality by DBAN

Treatment of cells with DBAN at 3–50 μM for 3 h did not cause changes in the histogram of PI fluorescence (Fig. 1A). Thus, DBAN administration at 50 μM or less did not change the percentage of dead cells or membrane-comprised cells in the population (Fig. 1B). However, cell treatment with 100 μM DBAN for 3 h significantly increased

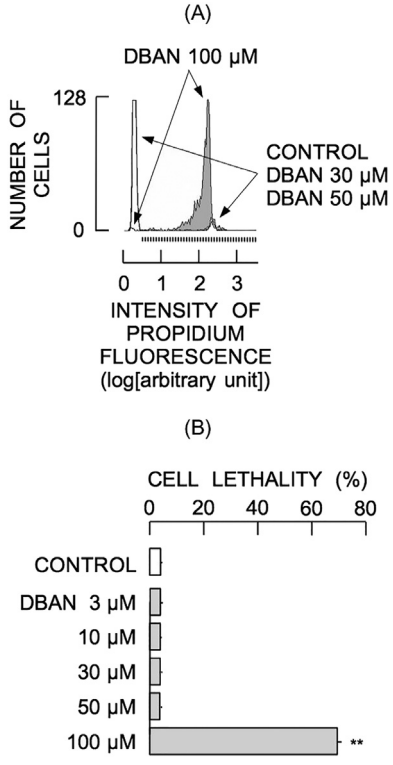

Fig. 1. DBAN-induced increase in cells exhibiting PI fluorescence (cell lethality). (A) Histograms of PI fluorescence recorded from cells treated with DBAN. Each histogram was constructed using 2500 cells. Cytogram is a representative one in eighteen measurements. (B) Concentration-response relationship for DBAN-induced changes in cell lethality. Columns and bars show mean ± standard deviation of six to eight samples. Each experimental series was performed thrice. Asterisks (**) indicate significant differences ($P < 0.01$) between the control group (CONTROL) and the group of cells treated with DBAN.