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Cytotoxic actions of 2,2-dibromo-3-nitrilopropionamide, a biocide in hydraulic fracturing fluids, on rat thymocytes

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Abstract

2,2-Dibromo-3-nitrilopropionamide (DBNPA) is a major biocide in hydraulic fracturing fluids. Most biocides in fracturing fluids are considered to have low acute toxicity to mammals, but little information is available in the literature regarding the toxic actions of DBNPA on mammalian cells. This information is important to suggest the DBNPA toxicity on wild mammals. In this study, the effects of DBNPA on rat thymocytes were studied using flow cytometric techniques in order to further characterize the cytotoxicity of DBNPA for its safe use. DBNPA at 3-7.5 μM produced a steep concentration-dependent increase in cell lethality. At 5 μM, DBNPA significantly depolarized membranes with disturbance of asymmetrical distribution of membrane phospholipids. The lethal effect of DBNPA was completely abolished under cold conditions, and was augmented in the presence of ethanol. It is suggested that the lethal action of DBNPA is linked to changes in membrane fluidity. Because the concentration-dependent change of DBNPA-induced lethal action was very steep under in vitro conditions, the adverse actions of DBNPA on wild mammals are concerning, even though such reports have not yet surfaced. (177 words)

Keywords:
2,2-dibromo-3-nitrilopropionamide; thymocytes; cytotoxicity; membrane fluidity; cell death

Introduction

There was a dramatic increase in production of natural gas and oil extracted from shale reservoirs over last decade[1]. This dramatic increase was aided by technical advances in hydraulic fracturing. Because shale gas and oil are trapped in rock, extraction is needed. Bacterial control by biocides is required in hydraulic fracturing operations in order to maintain the extraction by preventing biofilm formation at filters[2]. 2,2-Dibromo-3-nitrilopropionamide (DBNPA) is one of two major biocides used in hydraulic fracturing fluids[2,3], and does not have a measurable risk to the aquatic ecosystem[4]. Most biocides used in fracturing fluids are considered to have relatively low acute toxicity to mammals. The median lethal oral dose of DBNPA for rats has been reported as either 178 mg/kg[5] or 207 mg/kg[2]. There is a lack of information in the literature regarding the toxic actions of DBNPA on mammalian cells; such information is necessary to predict the influence of DBNPA on wild mammals. In this study, the effects of DBNPA on rat thymic lymphocytes were studied using flow cytometric techniques with appropriate fluorescent probes. We observed some unique actions of DBNPA at low micromolar concentrations and examined their possible mechanisms. This study may provide information for characterizing the cytotoxicity of DBNPA for its safe use.

Methods and Materials

Cell preparation

This study was approved by the Committee for Animal Experiments of Tokushima University, Tokushima, Japan (No. 14124). Experimental methods were similar to those described in previous papers[6,7]. The cell suspension was prepared as previously reported[7]. In brief, thymus glands dissected from ether-anesthetized rats were sliced under cold conditions. The slices were triturated in Tyrode's solution to dissociate the thymocytes. The cell suspension was incubated at 36-37°C for 1 h before the experiment.

Thymocytes were chosen because of the following reasons. First, the cells are dissociated without treatment with proteolytic enzymes that may compromise cell membranes. The cell viability of dissociated thymocytes under control conditions was greater than 95%. Secondly, thymocytes are suitable for applying to a flow cytometer because of their spherical shape, size, and homogeneity. Finally, thymus is a primary lymphoid organ, largest and most active during

the neonatal and pre-adolescent periods, of the immune system. Therefore, the thymus as a target for environmental pollutants is toxicologically interesting.

Chemicals

DBNPA was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The purity was >99%. Annexin V-FITC, propidium iodide, and bis-(1,3-dibutylbarbituric acid)trimethineoxonol (Oxonol) were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless otherwise mentioned.

Fluorescence measurements of cellular parameters

To assess cell lethality (percent population of dead cells) using propidium iodide, the dye was added to the cell suspension to a final concentration of 5 μM. Exposure of phosphatidylserine on the outer surface of cell membranes, a marker of early stage apoptosis, was detected using 10 μL/mL annexin V-FITC[8]. Oxonol (500 nM) was added to the cell suspension to assess the change in membrane potential. Oxonol fluorescence was measured from the cells that were not stained with propidium (living cells with intact membranes). Fluorescence of FITC and Oxonol was detected at 530 ± 20 nm. Propidium fluorescence was detected at 600 ± 20 nm. Fluorescence was measured and analyzed using a flow cytometer (CytoACE-150, JASCO, Tokyo, Japan)

WST-1 assay

Cells in a 96-well tissue culture plate were incubated with the WST-1 reagent for 2 h. After this incubation period, the formazan dye was quantitated with a microplate reader (MTP-310Lab, Corona Electric, Hitachinaka, Japan). The measured absorbance at 450 nm correlates with the number of viable cells.

Statistical analysis

Statistical analyses were performed by ANOVA with post-doc Tukey's multivariate analysis (Excel Toukei in Japanese, SSRI, Tokyo, Japan). P-values of less than 0.05 were considered significant. The results (including columns and bars in figures) were expressed as mean and standard deviation of four samples.

Results

DBNPA-induced increase in cell lethality

As shown in Fig. 1A, incubation with 5 μM DBNPA for 3 h increased the population of cells exhibiting propidium fluorescence (dead cells). The dose-response curve of the DBNPA-induced increase in cell lethality is summarized in Fig. 1B. Cell lethality was significantly increased by incubation with 5-7.5 μM DBNPA for 3 h, even to more than 90% in the case of 7.5 μM DBNPA. Thus, the dose-response relationship was very steep. Results were confirmed by WST-1 assay (Fig. 1C). The cell viability was significantly decreased by 5-7.5 μM DBNPA.

(Figure 1 near here)

DBNPA-induced increase in percent population of annexin V-positive living cells

Incubation with 5 μM DBNPA for 1 h also increased the population of cells exhibiting FITC fluorescence, but not propidium fluorescence (area A of Fig. 2A), when propidium iodide and annexin V-FITC were applied to cells, meaning that DBNPA increased the population of living cells with phosphatidylserine exposed on the outer membrane surface. Results were summarized as the DBNPA-induced change in cell population (Fig. 2B).

(Figure 2 near here)

DBNPA-induced augmentation of Oxonol fluorescence in living cells

As described above, incubation with 5 μM DBNPA disrupted the sequence of membrane phospholipids. It raised the possibility that DBNPA might have depolarized the membranes. To test this possibility, the change in intensity of Oxonol fluorescence by 1-5 μM DBNPA was examined. Incubation with 5 μM DBNPA, but not 1-3 μM, strongly augmented oxonol fluorescence in living cells. The intensity of oxonol fluorescence in the presence of 5 μM DBNPA indicated that the membranes of living cells were significantly depolarized.

(Figure 3 near here)

DBNPA-induced change in cell lethality under cold conditions

DBNPA changed the membrane distribution of phospholipids (Fig. 2). Various phospholipid species can exert an effect on membrane fluidity[9]. It was possible that membrane

fluidity was modified by DBNPA. In many preparations, membrane fluidity decreases with a decrease in temperature[10]. Therefore, the effect of DBNPA was examined under cold conditions. As shown in Fig. 4, under cold conditions (3-4°C) the lethal action of 7.5 μM DBNPA was completely attenuated. Warming the cell suspension from 3-4°C to 36-37°C produced a lethal effect on the cells. Thus, it is concluded that the lethal action of DBNPA is temperature-dependent. Precise analysis on the temperature-dependence will be performed in future study.

(Figure 4 near here)

Lethal action of DBNPA in the presence of ethanol

Since ethanol (20-320 mM) is reported to increase membrane fluidity in erythrocyte membranes[11], the change in cell lethality by 5 μM DBNPA was examined in the presence of 30--300 mM ethanol. Incubation with 30-300 mM ethanol for 2 h did not significantly change cell lethality. Simultaneous incubation with ethanol at concentrations of 100-300 mM for 2 h further augmented the lethal action of DBNPA in a concentration-dependent fashion; however, this was not the case for 30 mM ethanol (Fig. 5).

(Figure 5 near here)

Discussion

Cellular actions of DBNPA

From the results shown in Fig. 4 and 5, the cytotoxicity of DBNPA is hypothesized to be related to changes in membrane fluidity. DBNPA is electrophilic and probably reacts with nucleophilic sulfur-containing amino acids and amine-containing amino acids in membrane proteins[12,13]. These proteins that reside within the membrane structure affect fluidity[14]. It is likely that DBNPA modifies the structure of these proteins, resulting in changes in membrane fluidity. The lethal action of DBNPA was sensitive to experimental temperatures (Fig. 4); thus, the agent may be less toxic to poikilothermic animals under naturally cool or cold conditions.

DBNPA at concentrations of 3 μM or more (up to 10 μM) was observed to possess lethal action in a very steep concentration-dependent manner (Fig. 1). Incubation with 5 μM DBNPA disturbed the asymmetrical distribution of phospholipids in membranes (Fig. 2); the living cells lost membrane potential in the presence of 5 μM DBNPA (Fig. 3). Therefore, DBNPA is

hypothesized to be a membrane-active agent. In preliminary unpublished study, DBNPA at 1–3 μM slightly increased the intensity of Fluo-3 fluorescence, an indicator of intracellular Ca^{2+} level. Further studies on DBNPA-induced changes in membrane permeability and the intracellular concentration of Ca^{2+} will be necessary because an excessive increase in intracellular Ca^{2+} levels is linked to cell death[15].

Toxicological implication

DBNPA is used as a common electrophilic biocide at concentrations ranging from 0.0002 % to 0.02 % in paper mills, cooling water systems, heat exchangers, and laboratory equipment. DBNPA is also employed in fracturing fluids[16]. However, a portion of injected biocides is supposed to resurface as transformation product(s)[2]. DBNPA is degraded by hydrolysis[2]. DBNPA concentration may decrease after hydraulic fracturing[17]. Thus, the risks associated with biocides in fracturing fluids probably differ before and after hydraulic fracturing. If DBNPA were to be discharged into the aquatic ecosystem, the concentrations would be much lower than those used when it is used as a biocide. Under present in vitro conditions, the lethal concentrations of DBNPA in rat thymocytes were determined to be between 3-10 μM, which is equivalent to about 0.00007–0.00024 % (about 0.7-2.4 mg/L). Information on environmental DBNPA concentrations around shale gas and oil reservoirs is not available at present. Information on the concentrations of DBNPA in wild mammals and the pharmacokinetics of DBNPA in experimental animals is also unavailable. Therefore, it is difficult to predict the influence of DBNPA on the heath of wild mammals from the present in vitro results. Because the concentration-dependent change in DBNPA-induced lethality in rat thymocytes is steep (Fig. 1), the adverse actions of DBNPA on wild mammals could be of continuously concern even though such reports have not yet surfaced.

The profile of DBNPA toxicity is shown as US EPA Archive document[5]. In a subchronic toxicity study, rats were given DBNPA for 90 days by gavage at doses of 0, 5, 13, or 30 mg/kg/day. The level of no observed effect (NOEL) was 5 mg/kg/day. The lowest level of observed effect (LOEL) was 13 mg/kg/day. The potency of DBNPA cytotoxity seems to be less than those of tributyltin[18], triphenyltin[19], and 4,5-dichloro-2-octyl-4-isothiazolin-3-one[20]. Under our same experimental conditions, the lethal concentrations of organotin antifoulants in rat thymocytes were less than 1 μM[18,19]. Thus, the toxic action of DBNPA may not attract attention. However, it is reported that the hydrolysis products of DBNPA, dibromoacetic acid and dibromocetonitrile, can be more toxic and/or persistent[21]. In this aspect, further study will be

necessary.

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Conflict of interests

All authors affirm that there are no conflicts of interest to declare.

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Figure legends

Figure 1

DBNPA-induced change in the cell lethality of rat thymocytes. (A) Change in population of cells stained with propidium iodide at 1 h after incubation with 5 μM DBNPA. The dotted bars under the cytograms indicate the population of cells exhibiting propidium fluorescence. The cytogram was constructed with 2000 cells. (B) Concentration-dependent increases in cell lethality (percent population of cells exhibiting propidium fluorescence) at 1 h after incubation with DBNPA. The column and bar show the mean value and standard deviation of four samples, respectively. Asterisks (**) indicate significant differences ($P < 0.01$) between control group (CONTROL) and the group of cells treated with DBNPA. (C) DBNPA-induced changes in cell viability as estimated with WST assay at 2 h after incubation with DBNPA.

Figure 2

DBNPA-induced disturbance of asymmetrical distribution of membrane phospholipids before cell death. (A) DBNPA-induced change in cell population. The cell population was classified with propidium iodide and annexin V-FITC. The cells exhibiting neither propidium fluorescence nor FITC fluorescence were defined as intact living cells (INTECT LIVING CELLS, area N). The cells exhibiting FITC fluorescence but not propidium fluorescence were classified as living cells with phosphatidylserine exposed on the outer membrane surface (ANNEXIN V-POSITIVE LIVING CELLS, area A). The dead cells were stained with propidium iodide, and exhibited propidium fluorescence (DEAD CELLS, areas P and AP). The cytogram was constructed with 2000 cells. (B) Percent changes in cell population described above by incubation with 5 μM DBNPA. The column and bar show the mean value and standard deviation of four samples, respectively. Asterisks (**) indicate significant differences ($P < 0.01$) between the control group (CONTROL) and the group of cells treated with DBNPA.

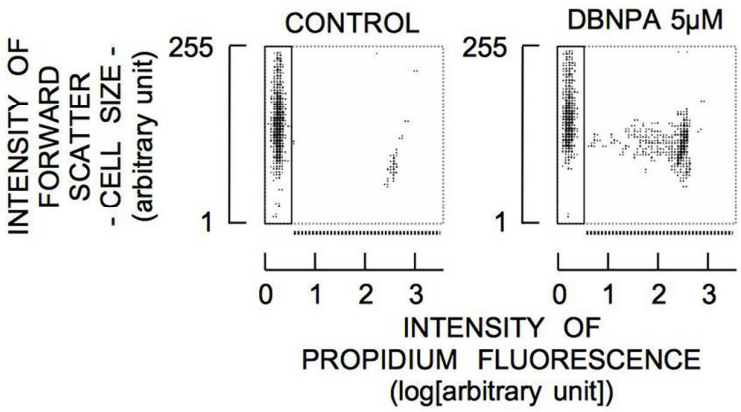
Figure 3

DBNPA-induced change in Oxonol fluorescence (membrane potential) of living cells. Cells were incubated with DBNPA for 1 h. The column and bar show the mean value and standard deviation of four samples, respectively. Asterisks (**) indicate significant differences ($P < 0.01$) between the control group (CONTROL) and the group of cells treated with DBNPA.

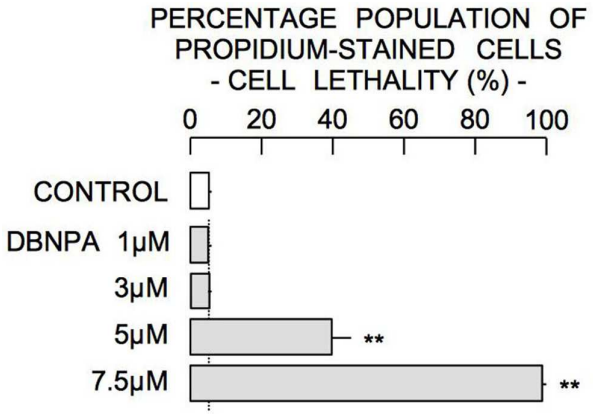
316 Figure 4
317 DBNPA-induced change in cell lethality under control (36-37°C) and cold (3-4°C) temperature
318 conditions. The column and bar show the mean value and standard deviation of four samples,
319 respectively. Asterisks (**) indicate significant differences ($P < 0.01$) between the control group
320 (CONTROL) and the group of cells treated with DBNPA.
321
322 Figure 5
323 Change in DBNPA-induced action in the absence and presence of ethanol (30-300 mM). The
324 column and bar show the mean value and standard deviation of four samples, respectively.
325 Asterisks (**) indicate significant differences ($P < 0.01$) between the control group
326 (CONTROL) and the group of cells treated with DBNPA. Symbols (##) show significant
327 differences between the groups of cells treated with DBNPA in the absence and presence of
328 30-300 mM ethanol.
329
330

Figure 1

A.


CONTROL
DBNPA 5μM
INTENSITY OF FORWARD SCATTER - CELL SIZE - (arbitrary unit)
255
1
0 1 2 3
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])

B.


PERCENTAGE POPULATION OF PROPIDIUM-STAINED CELLS - CELL LETHALITY (%) -
0 20 40 60 80 100
CONTROL
DBNPA 1μM
3μM
5μM
7.5μM
**
**

C.

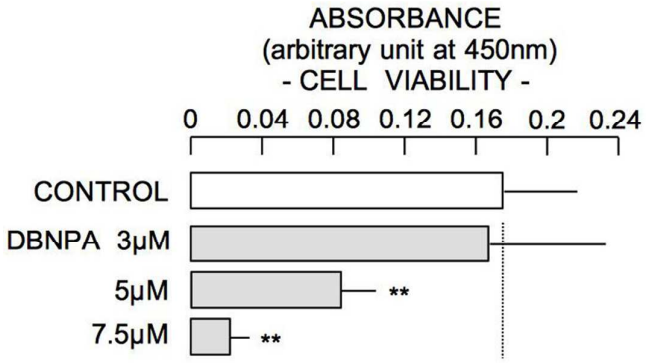
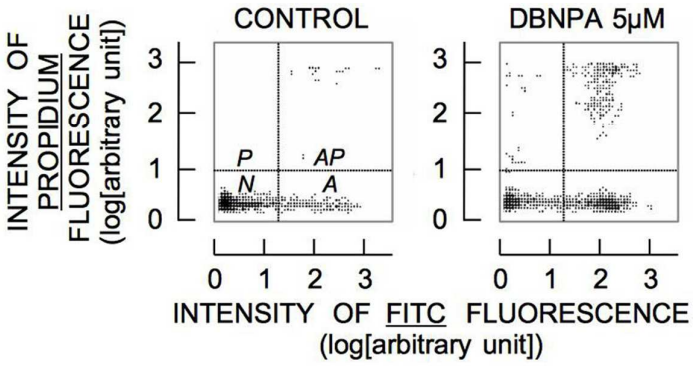

ABSORBANCE
(arbitrary unit at 450nm)
- CELL VIABILITY -
0
0.04
0.08
0.12
0.16
0.2
0.24
CONTROL
DBNPA 3μM
5μM
7.5μM
**
**

Figure 2

A.


CONTROL
DBNPA 5μM
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])
P
AP
N
A
0
1
2
3
INTENSITY OF FITC FLUORESCENCE (log[arbitrary unit])

B.

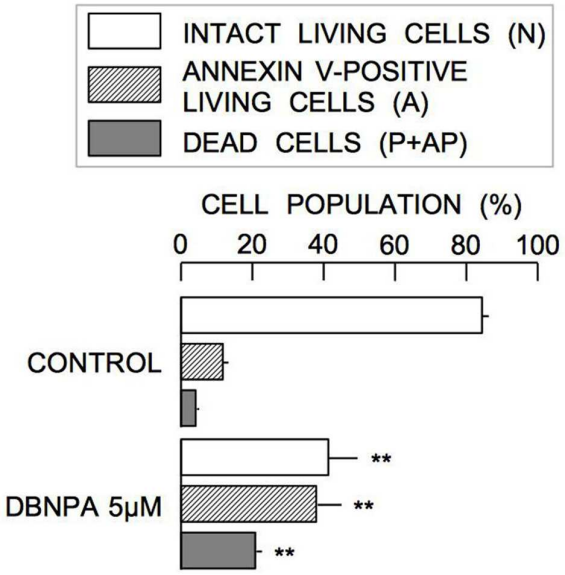

INTACT LIVING CELLS (N)
ANNEXIN V-POSITIVE LIVING CELLS (A)
DEAD CELLS (P+AP)
CELL POPULATION (%)
0
20
40
60
80
100
CONTROL
DBNPA 5μM
**
**
**

Figure 3

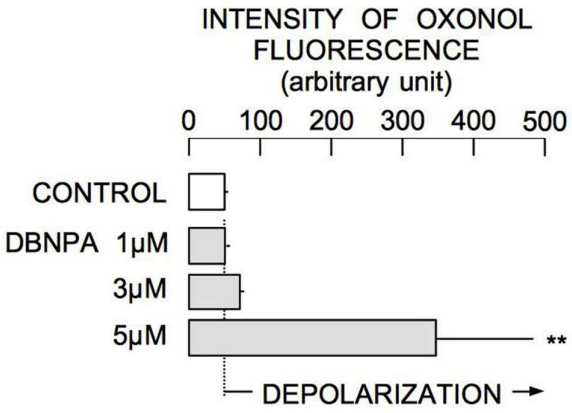

INTENSITY OF OXONOL FLUORESCENCE
(arbitrary unit)
0
100
200
300
400
500
CONTROL
DBNPA 1μM
3μM
5μM
**
DEPOLARIZATION

Figure 4

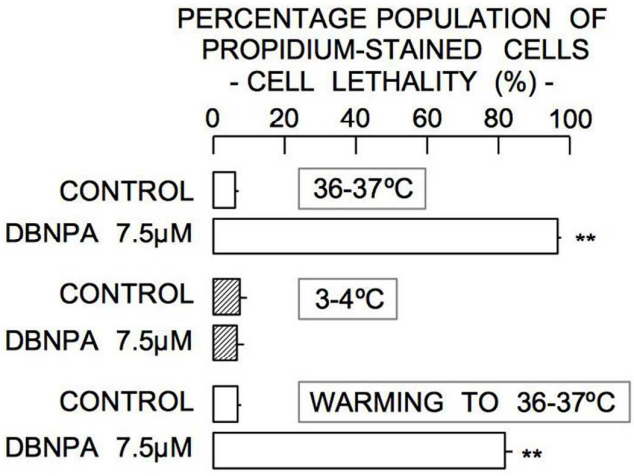
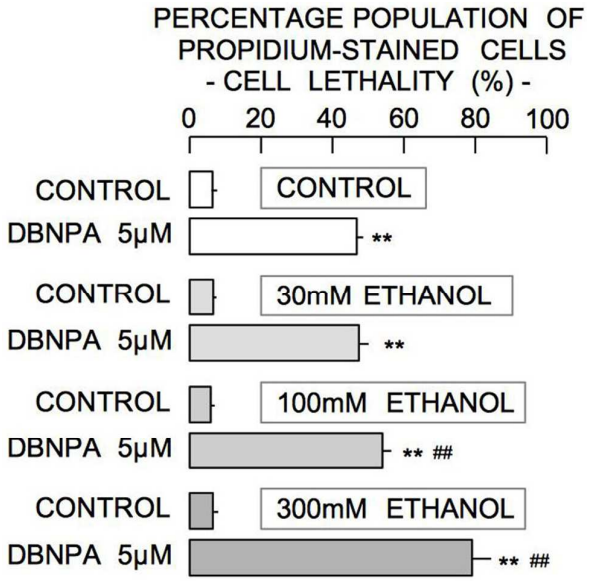

PERCENTAGE POPULATION OF
PROPIDIUM-STAINED CELLS
- CELL LETHALITY (%) -
0 20 40 60 80 100
CONTROL
36-37°C
DBNPA 7.5µM
**
CONTROL
3-4°C
DBNPA 7.5µM
CONTROL
WARMING TO 36-37°C
DBNPA 7.5µM
**

Figure 5


PERCENTAGE POPULATION OF
PROPIDIUM-STAINED CELLS
- CELL LETHALITY (%) -
0 20 40 60 80 100
CONTROL
CONTROL
DBNPA 5µM
**
CONTROL
30mM ETHANOL
DBNPA 5µM
**
CONTROL
100mM ETHANOL
DBNPA 5µM
** ##
CONTROL
300mM ETHANOL
DBNPA 5µM
** ##