Original Article

Study of the Toxicity of 1-Bromo-3-Chloro-5,5-Dimethylhydantoin to Zebrafish*

LI WanFang, WEI JinFeng, JIN HongTao, HUANG MingFang, ZHANG JingXuan, LI ChengHe, CHEN ChaoJie, LIU Chang, and WANG AiPing[#]

Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

Abstract

Objective 1-Bromo-3-chloro-5,5-dimethylhydantoin (BCDMH) is a solid oxidizing biocide for water disinfection. The objective of this study was to investigate the toxic effect of BCDMH on zebrafish.

Methods The developmental toxicity of BCDMH on zebrafish embryos and the dose-effect relationship was determined. The effect of BCDMH exposure on histopathology and tissue antioxidant activity of adult zebrafish were observed over time.

Results Exposure to 4 mg/L BCDMH post-fertilization was sufficient to induce a number of developmental malformations, such as edema, axial malformations, and reductions in heart rate and hatching rate. The no observable effects concentration of BCDMH on zebrafish embryo was 0.5 mg/L. After 96 h exposure, the 50% lethal concentration (95% confidence interval (CI)) of BCDMH on zebrafish embryo was 8.10 mg/L (6.15-11.16 mg/L). The 50% inhibitory concentration (95% CI) of BCDMH on hatching rate was 7.37 mg/L (6.33-8.35 mg/L). Histopathology showed two types of responses induced by BCDMH, defensive and compensatory. The extreme responses were marked hyperplasia of the gill epithelium with lamellar fusion and epidermal peeling. The histopathologic changes in the gills after 10 days exposure were accompanied by significantly higher catalase activity and lipid peroxidation.

Conclusion These results have important implications for studies on the toxicity and use of BCDMH and its analogs.

Key words: Developmental toxicity; Histopathological effects; 1-Bromo-3-chloro-5,5-dimethylhydantoin (BCDMH)

Biomed Environ Sci, 2011; 24(4):383-390 doi:10.3967/0895-3988.2011.04.009 ISSN:0895-3988

www.besjournal.com(full text) CN: 11-2816/Q

INTRODUCTION

1-Bromo-3-chloro-5,5-dimethylhydantoin (BCDMH) is a solid oxidizing biocidal product utilized for water disinfection. It is widely used to sterilize swimming pools, tap water, aquaculture facilities, and public places[1-3]. When a hydrotherapy physiotherapist presented with irritant contact dermatitis[4], subsequent investigation revealed that the likely causative agent was BCDMH, which was used to disinfect the hydrotherapy pool. This case illustrated the problems associated with

*This work was supported by the National Science and Technology Major Project "Creation of Major New Drugs", 2008ZX09305-001.

#Correspondence should be addressed to WANG AiPing. Tel: 86-10-83169349. Fax: 86-10-83169038. E-mail: wangaiping@imm.ac.cn

Biographical note of the first author: LI WanFang, female, born in 1983, Ph. D candidate, majoring in pharmacology and toxicology.

Received: February 24, 2011; Accepted: April 28, 2011

prolonged BCDMH exposure. The use of BCDMH in the UK and North America has produced numerous cases of rashes, most of which were transient, but some were serious (especially after re-exposure). In spite of this, toxicological reports about BCDMH are rare. A comprehensive examination of its toxicity is necessary to ensure the safe use of BCDMH.

The objective of this study was to obtain more detailed information on the toxicity of BCDMH by examining its effects in zebrafish (*Danio rerio*), one of the most widely used fish models in aquatic toxicology. The study examined the developmental and histopathological responses of zebrafish after BCDMH exposure. Two experiments were carried out. The first determined the developmental toxicity of BCDMH in zebrafish embryo in terms of the major toxic effects, and the dose-effect relationship. The second experiment was designed to detect pathological and tissue antioxidant changes over time after BCDMH exposure. To our knowledge, no such studies on BCDMH have been conducted. The present study aims to provide more toxicological information for the safe use of BCDMH.

Considering that BCDMH is mainly used for water treatment, aquatic creatures are most suitable for the study of its toxicological effects. The zebrafish has already been used as a model organism in numerous studies to assess the toxicity endpoints of compounds or their mechanisms of action[5-11]. The zebrafish offers several advantages for toxicity testing, including economic husbandry requirements, high fecundity, rapid external development, transparency during organogenesis, and an identifiable diploid genome[12]. The gills and body surfaces are in direct contact with the external environment, promoting the exchange of gases and ionic balance[13-14]. Gills are also sensitive to the acute effects of exposure to toxic compounds, as they are quick to react to unfavorable environmental conditions. According to Hinton et al., histopathologic changes reflect a higher-level response, resulting from prior alterations in physiologic and/or biochemical functions[15]. Histopathologic changes are sensitive measurement endpoints.

MATERIALS AND METHOD

Zebrafish Husbandry

Adult wild-type zebrafish of different sexes were purchased from a local pet supply store and housed in a zebrafish aquatic housing system (Aquaneering®, CA, USA). The facility used dechlorinated municipal water. All physicochemical water parameters were maintained at optimal conditions for zebrafish (26-28°C, dissolved oxygen>60%, pH 6.5-8, ratio of light to dark time 14:10, unionized ammonia <0.01 ppm). The fish were fed once a day with freeze-dried brine shrimp (Rainone®, Beijing, China), and were allowed to acclimatize for 2 weeks before the experiment. Less than 1% of the population died during acclimatization. All procedures were approved by the Animal Care and Welfare Committee Institute of Materia Medica, CAMS & PUMC.

Test Solutions

BCDMH (CAS number 16079-88-2, $C_5H_6BrClN_2O_2$, 96% whitish crystal powder) was obtained from Sani-Marc Inc. (Oakville, Ontario, Canada). Dosing was performed by suspending dry BCDMH powder in Milli-Q water, with 15 min ultrasonication, and adding the requisite volumes of stock solution to each experimental solution.

Developmental Toxicity

Three independent experiments (24 embryos per treatment) were conducted to determine dose-dependent effects of BCDMH on zebrafish embryo mortality, and endpoints of BCDMH toxicity. The test concentrations were selected based on the 0% to 100% effect levels, as derived from a range finding test. Stock solutions of BCDMH were diluted to 16.0, 8.0, 4.0, 2.0, 1.0, and 0.5 mg/L. Standardized water was used as the negative control.

Prior to spawning, males and females were separately housed for a minimum of 5 days. The day before eggs were required, males and females were placed in breeding tanks with a 2:1 male:female ratio. The fish were left undisturbed overnight and eggs were collected 1 h after exposure to light the next morning. Immediately after egg collection, the embryos were exposed to the test media containing different BCDMH concentrations within 2 h post-fertilization (hpf). At 4 hpf, fertilization success was determined and 24 fertilized eggs for each concentration were individually transferred into a 24-well plate (Costar, Corning Incorporated, USA) containing 2 mL solution, with one embryo per well. The plates were covered with parafilm. The embryos were placed in a temperature- and light-controlled incubator set to the same circadian rhythm as the adult zebrafish. The endpoints used for assessing developmental toxicity included embryonic survival, hatching rate, heart rate (visually determined as the mean for 10 embryos, counted for 30 s), edema, and skeletal deformity. These characteristics were

described for the embryos and larvae from both the control and the treated groups.

Exposure of Adult Zebrafish to BCDMH

Concentrations of BCDMH were chosen according to a previous study, which observed slight pathological gill changes after 96 h exposure to 0.7 mg/L BCDMH. Zebrafish were exposed to the control water and 0.7 mg/L test solution. The solution was changed daily. Tricaine (3-aminobenzoic acid ethylester, pH 7.0; Green Fortune, China) at 320 μg/mL was used to kill the zebrafish. The fish (8 per group) were killed after 1, 4, 7, and 10 days exposure for histopathologic analysis. After 10 days exposure, other fish (8 per group) were killed for gill and skeletal muscle samples, which were collected and frozen immediately in liquid N_2 for further analysis.

Histopathology

Fish were fixed by immersion in 10% buffered formalin for 24 h at 4 °C, washed twice in phosphate-buffered saline, and transferred to 75% ethanol until processing. After dehydration and embedding in paraffin wax, the fish were step-sectioned to obtain longitudinal sections. The sections were stained with hematoxylin and eosin, and examined under the ×20 objective of a Nikon Eclipse 80i microscope. Observed lesions were classified in three different progressive stages of severity following the classification by Poleksić and Mitrović-Tutundžić[13]. Gill injuries were manually counted in each slide. For semi-quantitative analysis, a value corresponding to absence (0), low presence (1), and high presence (2) was assigned to each injury[16].

Biochemical Analysis

After 10 days BCDMH exposure, gills and skeletal muscle samples were collected and immediately frozen in liquid N_2 for further analysis. Samples were homogenized in saline, and the homogenates were centrifuged at 10 000 *g* for 10 min at 4 °C using a 3K18 centrifuge (Sigma, Germany) to precipitate the insoluble material. The supernatants were tested for total protein and malondialdehyde (MDA) content, catalase (CAT) and superoxide dismutase (SOD) activity, as well as oxygen radical absorbance capacity (ORAC) and trolox equivalent antioxidant capacity (TEAC)[17-18]. All reaction mixtures were performed in duplicate.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 11.5; SPSS Company, Chicago, MI). All data were expressed as mean ± standard deviation (SD). For the developmental toxicity test, after 96 h exposure, the 50% lethal dose (LC_{50}) and the respective 95% confidence intervals (CI) were determined by probit analysis. For comparison between two groups, the significance of differences between means was determined by the Student *t*-test. Analysis of variance was used for multiple comparisons, followed by Dunnett's test. The difference among groups was significant at $P<0.05$, and highly significant at $P<0.01$.

RESULTS

Developmental Toxicity

Concentration-response curves were created for mortality, edema, axial malformation, hatching and heart rate of the zebrafish (Figures 1-3). In addition, eye morphogenesis retardation and weak pigmentation were observed in groups exposed to 4.0 and 8.0 mg/L BCDMH (data not shown). Only embryos exposed to higher concentrations of 4.0 and 8.0 mg/L BCDMH showed significant and concentration-dependent reductions in mean heart rate (Figure 3). The no observable effects concentration of BCDMH on the zebrafish embryo was 0.5 mg/L. After 96 h exposure, the LC_{50} (95% CI) of BCDMH was 8.10 mg/L (6.15-11.16 mg/L). The 50% inhibitory concentration (95% CI) of BCDMH on the hatching rate was 7.37 mg/L (6.33-8.35 mg/L). The main characteristics of axial malformations were curled axes and local expansion of the notochord. At 0.5 mg/L, no detectable developmental abnormalities were observed. From 1.0 mg/L, however, weak to

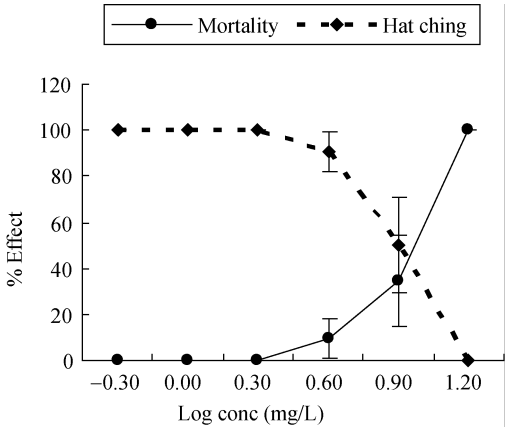

Figure 1. Concentration-response curve for mortality and hatching after BCDMH exposure (n=3) at 96 hpf. The % effect ($\bar{x} \pm s$) is shown versus the logarithm of the concentrations (log mg/L).

severe developmental defects were observed according to concentration (Figure 4).

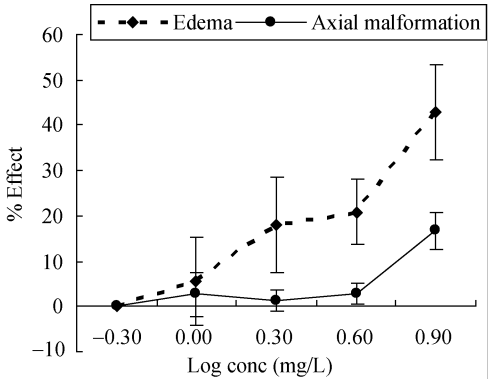

Figure 2. Concentration-response curve for edema and axial malformation for BCDMH exposure (n=3) at 96 hpf. The % effect ($\bar{x} \pm s$) is shown versus the logarithm of the concentrations (log mg/L).

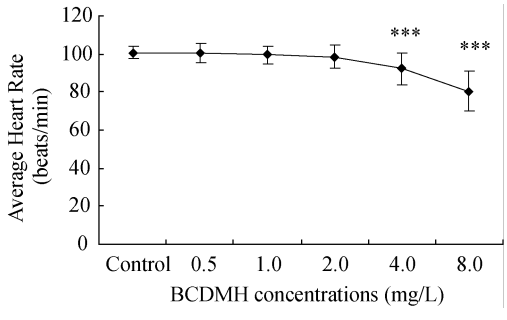

Figure 3. Average heart rate among zebrafish embryos after 48 h of exposure. A significant reduction in heart rate occurred at higher concentrations of 4.0 and 8.0 mg/L BCDMH ($^{***}P$<0.001).

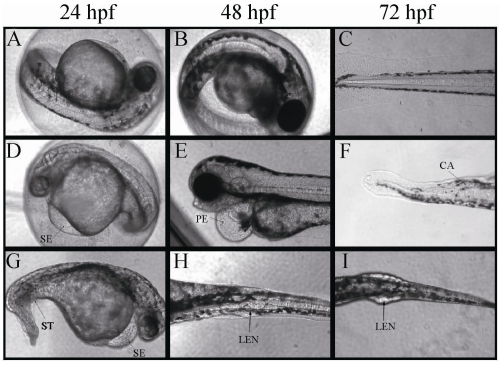

Figure 4. Normal (A-C) and abnormal (D-I) development of zebrafish embryos exposed to BCDMH for different periods (×100). CA, curled axis; LEN, local expansion of the notochord; PE, pericardial edema; SE, yolk sac edema; ST, shortened tail.

Histopathology

The adult fish in the control group had gills with normal morphology, i.e., secondary lamellae with distinguishable pillar cells and erythrocytes with well-defined spaces, normal development of the cartilaginous support in the respiratory area, and normal placement of the stratified epithelium in the gill filament. The fish exposed to 0.7 mg/L BCDMH showed signs of gill lesions (Figure 5, Table 1). The lesions found on the gills of the fish exposed to BCDMH became more accentuated with prolonged exposure, as shown by the more intense proliferations and second-stage alterations on the 7th and 10th days.

Table 1. Histological Alterations Observed in Gills of Zebrafish Exposed to 0.7 mg/L BCDMH

Gill Lesions	Stage	1 d		4 d		7 d		10 d	
		C	E	C	E	C	E	C	E
Lifting of Respiratory Epithelial Cells	I	1	1	0	1	0	2	1	2
Decreased Interlamellar Space	I	0	0	0	1	0	2	0	2
Hyperplasia from the Base to Approximately Half the Length of the Secondary Lamellae	I	0	0	0	1	0	2	0	2
Fusion of the Tips of the Secondary Lamellae	I	0	0	0	1	0	2	0	2
Fusion of Several Secondary Lamellae	I	0	0	0	1	0	2	0	2
Lamellar Telangiectasia	I	0	0	0	1	0	1	0	1
Rupture and Peeling of the Lamellar Epithelium	II	0	0	0	0	0	1	0	1
Complete Fusion of All the Secondary Lamellae	II	0	0	0	0	0	0	0	2
Total		1	1	0	6	0	13	1	17

Note. C, Control group; E, BCDMH exposure group. A value corresponding to absence (0), low presence (1), and high presence (2) was assigned to each injury. An importance factor of 1 or 2 was assigned to each lesion based on the level.

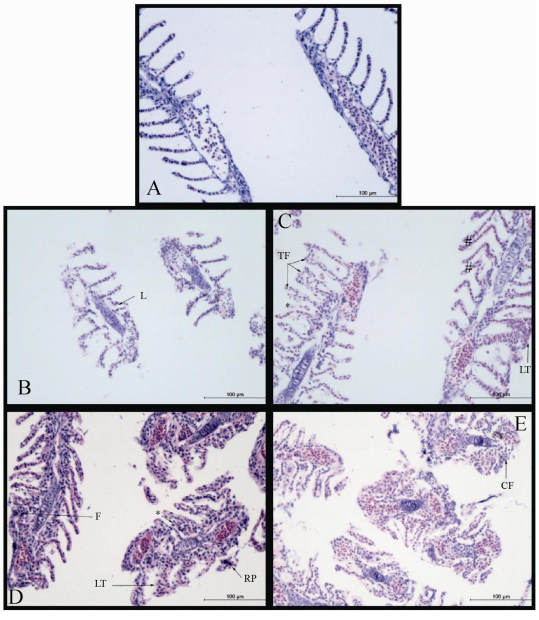

Figure 5. Representative sections of gills after 1, 4, 7, and 10 days BCDMH exposure. A) Control; B) After 1 day exposure; C) After 4 days exposure; D) After 7 days exposure; E) After 10 days exposure. Filament epithelium hyperplasia (*); Lifting of respiratory epithelial cells (L); lamellar telangiectasia (LT); decreased interlamellar space (#); fusion of the tips of secondary lamellae (TF); fusion of several secondary lamellae (F); complete fusion of all secondary lamellae (CF); rupture and peeling of epithelium (RP).

The normal epidermis of zebrafish is composed of keratinocytes, mucous cells, and club cells. Keratinocytes are squamous epithelial cells, slightly smaller than club cells, interconnected by desmosomes. Mucous cells have nuclei pushed to one side. Club cells are pink, with centrally located nuclei and occasionally have scalloped edges. The epidermis exposed to BCDMH (Figure 6, Table 1) became thinner than normal. The epidermis even peeled with prolonged exposure (on the 10th day). The most marked characteristic was mucous cell hyperplasia, increased mucus secretion, and appearance of vacuolated mucous cells.

Biochemical Analysis

Antioxidant responses are presented in Table 2. Compared with the control, significantly higher CAT activity was observed in the gills and muscles (P<0.05) after 10 days exposure of adult zebrafish to 0.7 mg/L BCDMH. MDA content was significantly higher (P<0.01) in the gills after 10 days BCDMH exposure.

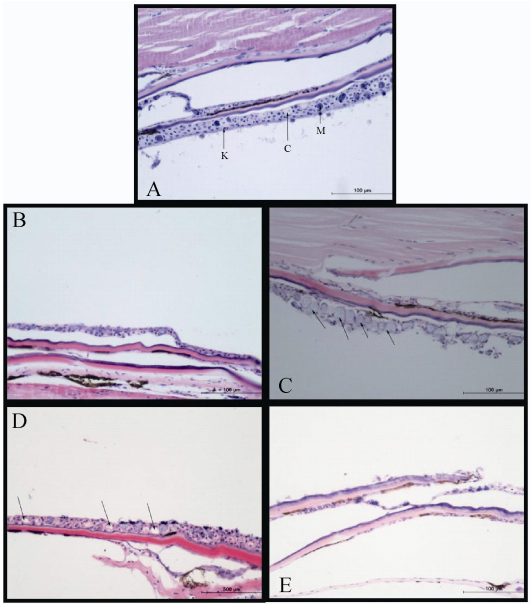

Figure 6. Representative sections of epidermis after 1, 4, 7, and 10 days BCDMH exposure. A) Control: keratinocytes (K), mucous cells (M), club cells (C). B) After 1 day exposure, the epidermis became thinner than normal. C) After 4 days exposure: hyperplasia of mucous cells (arrows); D) After 7 days exposure: vacuolated mucous cells (arrows). E) After 10 days exposure: peeling of the epidermis.

Table 2. Effect of 10 d Exposure to 0.70 mg/L BCDMH on Antioxidant Activity ($\overline{x} \pm s$)

Item	Gill		Muscle	
	Control	0.7 mg/L BCDMH	Control	0.7 mg/L BCDMH
ORAC (μmol Trolox/mg protein)	1.23 ± 0.30	1.05 ± 0.18	0.72 ± 0.19	0.66 ± 0.12
ABTS (μmol Trolox/mg protein)	0.66 ± 0.14	0.56 ± 0.06	0.39 ± 0.06	0.35 ± 0.03
SOD (U/mg protein)	0.63 ± 0.45	0.41 ± 0.20	0.17 ± 0.03	0.19 ± 0.04
CAT (U/mg protein)	2.51 ± 1.38	4.28 ± 2.04*	3.45 ± 1.35	3.61 ± 0.78*
MDA (nmol/mg protein)	8.44 ± 2.59	12.05 ± 9.31**	5.79 ± 2.34	6.64 ± 2.90

Note. * P<0.05, ** P<0.001.

DISCUSSIONS

Prior to this study, there have been no studies evaluating the developmental toxicity and histopathological responses of zebrafish to BCDMH.

Our results showed that exposure to 4 mg/L BCDMH post-fertilization was sufficient to induce a number of developmental malformations. Edema (pericardium and yolk sac), reduced heart rate, and axial malformation were the most marked effects. Related to heart failure was the presence of edema in the affected embryos. The associated decrease in blood flow could result from pericardial edema[19], as well as weak myocardial contraction[20]. Disruption of the central nervous system (CNS) and inhibition of acetylcholinesterase are suspected to play roles in heart rate abnormalities[21-22].

Axial malformations were defined as developmental abnormalities associated with the longitudinal axis, including a hooked, curved, curled, or clubbed axis[23].The notochord is the primary axial structure upon which many other tissues depend for their proper formation and differentiation. Toxicants that disrupt normal notochord morphogenesis and differentiation may therefore result in permanent skeletal deformities, muscle abnormalities, and neurological dysfunction. A study in rainbow trout embryos revealed that a number of dithiocarbamates were teratogenic, with the notochord being particularly sensitive[24]. Lien et al. suggested that the notochord may become malformed by overactive muscle spasms[25]. Teraoka et al. recently proposed a similar explanation for notochord waviness following 24 h exposure to dithiocarbamate thiuram[26]. In their study, notochord malformations were prevented by inhibition of spontaneous muscle contractions by co-exposure to the anesthetic MS-222.

Reduced or abnormal pigmentation has also been reported in both zebrafish and *Xenopus laevis* embryos after exposure to solvents and related chemicals[27-29]. Whether such effects are mediated through the CNS or result from direct action on melanocytes is still unclear. Finally, Fujii asserted that the pigment cells in most teleost fish undergo pigment aggregation or dispersion in response to environmental factors, including light and other physical or chemical factors[30].

The literature indicates that a variety of aquatic toxicants can affect gill structure and function. Gill pathologies in fish are common symptoms of the toxic effects of a wide variety of aquatic pollutants, including organophosphates, carbamates, miscellaneous herbicides, acidification, nitrogenous compounds, and heavy metal salts[31-32].

Studies of the morphologic changes induced by BCDMH show two types of responses: defense (inflammatory, lifting, lamellar fusion) and compensatory (cell proliferation)[33]. Both responses help bar the entry of toxicants and prevent them from reaching the bloodstream and, to a lesser extent, prevent the damage caused by the direct effects of BCDMH. An extreme response in the gills is marked epithelial hyperplasia with fusion of adjacent lamella and obliteration of the interlamellar space. Lamellar fusion can lead to pronounced reductions in respiratory surface area because of the disappearance of the secondary lamellae. Therefore, gas exchange and other gill functions are hindered[34].

Lamellar vasodilatation was also found in zebrafish exposed to BCDMH. Garcia-Santos et al. inferred that this lesion can induce changes in pillar cell structure, with consequent loss of their support function, and is probably responsible for the emergence of lamellar aneurysms in fish exposed to cadmium[35]. In another case, damaged pillar cells result in increased blood flow into the lamellae, causing dilation of the marginal channels, blood congestion, or even aneurysm[33,36]. Similar results are observed in Lates calcarifer exposed to cadmium[37]. However, Mallat suggested that these lesions are rarely associated with metal exposure[38].

The significant pathological alterations in the epidermis were mainly attributed to degenerative alterations. Degenerative changes, such as epidermal peeling, were common after 10 days of exposure. Lesions of this type are believed to reflect the direct deleterious effects of irritants rather than a compensatory response to pollutants.

Oxidative substances in cells may trigger an increase in antioxidant enzymes as a defense mechanism. SOD and CAT have related functions[39], and are always considered the first line of defense against oxygen toxicity due to their inhibitory effects on oxyradical formation[40]. SOD decomposes the superoxide anion into hydrogen peroxide and oxygen, whereas CAT acts as a scavenger for H_2O_2[41]. The results of this study are comparable with those of previous studies. No significant differences in SOD activity were observed between the BCDMH exposed groups and the control group. This verifies that BCDMH has little effect on superoxide anion metabolism under the experimental conditions tested. The results indicate that BCDMH markedly affected CAT activity. The MDA content in the gills

significantly increased, whereas it was not detected in skeletal muscle. Therefore, repeated exposure to BCDMH could result in a significant increase in lipid peroxidation in the gills of zebrafish.

CONCLUSIONS

Our results indicated that exposure to 4 mg/L BCDMH post-fertilization was sufficient to induce a number of adverse developmental effects, such as edema, axial malformations, and reduction in heart rate and hatching rate. In the 0.5 mg/L BCDMH group, there was no detectable developmental difference. Histopathologic analysis showed two types of responses induced by BCDMH, defensive and compensatory. The extreme responses were marked hyperplasia of the gill epithelium with lamellar fusion, and epidermal peeling. The histopathologic gill changes were accompanied by higher CAT activity and lipid peroxidation. These results have important implications in the use of BCDMH and its analogs. In practical applications, attention should be given to dose reduction and shortening of the disinfection cycles. Further investigations of the effects of BCDMH are currently underway in our laboratory.

REFERENCE

1. Beihoffer J, Bour E, Lowry J, et al. Identification and determination of the isomeric bromo-and/or chloro-substituted 1, 3-dihalo-5, 5-dimethylhydantoins used in disinfectants and molluscicides. J AOAC Int, 1996; 79(4), 823-8.
2. Brycki B, Seifert K, Szymanska K, et al. The effect of oxidizing biocides on desulfurication and denitrification processes. Pol J Environ Stud, 2000; 9(5), 363-7.
3. Donohoe H, Mattner S, Brett R, et al. Status of methyl bromide phase-out in the temperate australian strawberry industry. In: Proc. Annu. Intl. Res. Conf. on MB Alts. and Emiss. Red. 5-9 November, San Diego, USA. 2001; pp 41-4.
4. Loughney E, Harrison J. Irritant contact dermatitis due to l-bromo-3-chloro-5, 5-dimethylhydantoin in a hydrotherapy pool. Risk assessments, the need for continuous evidence-based assessments. Occupational Medicine, 1998; 48(7), 461-3.
5. Berghmans S, Hunt J, Roach A, et al. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res, 2007; 75(1), 18-28.
6. Chiu L, Cunningham L, Raible D, et al. Using the zebrafish lateral line to screen for ototoxicity. J Assoc Res Otolaryngol, 2008; 9(2), 178-90.
7. Hill A, Teraoka H, Heideman W, et al. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci, 2005; 86(1), 6-19.
8. Richards F, Alderton W, Kimber G, et al. Validation of the use of zebrafish larvae in visual safety assessment. J Pharmacol Toxicol, 2008; 58(1), 50-8.
9. Spitsbergen J, Kent M. The state of the art of the zebrafish model for toxicology and toxicologic pathology research-advantages and current limitations. Toxicol Pathol, 2003; 31(1), 62-87.
10. Tran T, Sneed B, Haider J, et al. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. Cancer Res, 2007; 67(1), 11386-92.
11. Winter M, Redfern W, Hayfield A, et al. Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs. J Pharmacol Toxicol, 2008; 57(3), 176-87.
12. Kimmel C, Ballard W, Kimmel S, et al. Stages of embryonic development of the zebrafish. Dev Dynam, 1995; 203(3), 253-310.
13. Poleksic V, Mitrovic-Tutundzic V. 1994. Sublethal and Chronic Effects of Pollutants on Freshwater Fish (R. Muller and R. Lloyd, Eds). Cambridge Univ. Press, Cambridge, UK.
14. Tkatcheva V, Hyv rinen H, Kukkonen J, et al. Toxic effects of mining effluents on fish gills in a subarctic lake system in NW Russia. Ecotox Environ Safe, 2004; 57(3), 278-89.
15. Hinton DE, Baumann P, Gardner G, et al. 1992. Histopathologic biomarkers. Lewis Publishers, Boca Raton, FL.
16. Sensini C, Della Torre C, et al. First observations of histopathological effects of 2, 4, 6-trinitrotoluene (TNT) in gills of European eel Anguilla anguilla (Linnaeus, 1758), histopathological effects of 2, 4, 6-trinitrotoluene in gills of European eel. Cell Biol Toxicol, 2008; 24(6), 621-8.
17. Ou B, Hampsch-Woodill M, Prior R. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem, 2001; 49(10), 4619-26.
18. Ukeda H, Sarker A, Kawana D, et al. Flow-injection assay of superoxide dismutase based on the reduction of highly water-soluble tetrazolium. Anal Sci, 1999; 15(4), 353-7.
19. Billiard S, Querbach K, Hodson P. Toxicity of retene to early life stages of two freshwater fish species. Environ Toxicol Chem, 1999; 18(9), 2070-7.
20. Couillard C. A microscale test to measure petroleum oil toxicity to mummichog embryos. Environ Toxicol, 2002; 17(3), 195-202.
21. Baratti C, Rubio M, Perec C, et al. Effect of chronic alcohol feeding on adrenergic and cholinergic neurotransmission mechanism. Am J Gastroenterol, 1980; 73(1), 21-7.
22. El-Mas M, Abdel-Rahman A. Effects of chronic ethanol feeding on clonidine-evoked reductions in blood pressure, heart rate, and their variability, time-domain analyses. J Pharmacol Exp Ther, 2003; 306(1), 271-8.
23. Reimers MJ, Flockton AR, Tanguay RL. Ethanol-and acetaldehyde-mediated developmental toxicity in zebrafish. Neurotoxicol Teratol 2004, 26(6), 769-81.
24. Van Leeuwen C, Helder T, Seinen W. Aquatic toxicological aspects of dithiocarbamates and related compounds. IV. Teratogenicity and histopathology in rainbow trout (Salmo gairdneri). Aquat Toxicol, 1986; 9(2-3), 147-59.
25. Lien N, Adriaens D, Janssen C. Morphological abnormalities in African catfish (Clarias gariepinus) larvae exposed to malathion. Chemosphere, 1997; 35(7), 1475-86.
26. Teraoka H, Urakawa S, Nanba S, et al. 2004. Notochord distortion by thiruram and other dithiocarbamate in zebrafish embryo. In Society of Toxicology, 43rd Annual Meeting. Baltimore, MD.
27. Dresser T, Rivera E, Hoffmann F, et al. Teratogenic assessment of four solvents using the Frog Embryo Teratogenesis Assay--Xenopus (FETAX). J Appl Toxicol, 1992; 12(1), 49-56.
28. Hen C, Cheng S. Cadmium affects muscle type development and axon growth in zebrafish embryonic somitogenesis. Toxicol Sci, 2003; 73(1), 149-59.

29. Lockwood B, Bjerke S, Kobayashi K, et al. Acute effects of alcohol on larval zebrafish, a genetic system for large-scale screening. Pharmacol Biochem Behav, 2004; 77(3), 647-54.
30. Fujii R. The regulation of motile activity in fish chromatophores. Pigment Cell Res, 2000; 13(5), 300-19.
31. Gupta A, Rajbanshi V. Mercury poisoning, Architectural changes in the gill of Rasbora daniconius(Hamilton). J Environ Biol, 1995; 16(1), 33-6.
32. Vijayalakshmi S, Tilak K. Effect of pesticides on the gill morphology of Labeo rohita. J Ecotoxicol Environ Monit, 1996; 6(1), 59-64.
33. Takashima F, Hibiya T. 1995. An Atlas of Fish Histology. Normal and Pathological Features, 2nd ed. Kodansha, Tokyo.
34. Rajbanshi V, Gupta A. Alterations in the architecture of the gill surface produced by water-borne copper in Heteropneustes fossilis (BLOCH). Acta Hydroch Hydrob, 1988; 16(3), 325-31.
35. Garcia-Santos S, Fontaínhas-Fernandes A, Wilson J. Cadmium tolerance in the Nile tilapia (Oreochromis niloticus) following acute exposure, assessment of some ionoregulatory parameters. Environ Toxicol, 2006; 21(2), 33-46.
36. Rosety-Rodriguez M, Ordo ez F, Rosety M, et al. Morpho-histochemical changes in the gills of turbot, Scophthalmus maximus L., induced by sodium dodecyl sulfate. Ecotoxicol Environ Saf, 2002; 51(3), 223-8.
37. Thophon S, Kruatrachue M, Upatham E, et al. Histopathological alterations of white seabass, Lates calcarifer, in acute and subchronic cadmium exposure. Environ Pollut, 2003; 121(3), 307-20.
38. Mallatt J. Fish gill structural changes induced by toxicants and other irritants, A statistical review. Can B Fish Aquat Sci, 1985; 42(4), 630-48.
39. Pandey S, Parvez S, Sayeed I, et al. Biomarkers of oxidative stress, a comparative study of river Yamuna fish Wallago attu (Bl. & Schn.). Sci Total Environ, 2003; 309(1-3), 105-15.
40. Van der Oost R, Beyer J, Vermeulen N. Fish bioaccumulation and biomarkers in environmental risk assessment, a review. Environ Toxicol Phar, 2003; 13(2), 57-149.
41. Gate L, Paul J, Ba G, et al. Oxidative stress induced in pathologies, the role of antioxidants. Biomed Pharmacother, 1999; 53(4), 169-80.