Gill histopathology in zebrafish model following exposure to aquacultural disinfectants

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Abstract

The effect of acute exposure of four disinfectants commonly used in aquacultural practice (formalin, potassium permanganate, benzalkonium chloride and malachite green) was studied on the histological structure of adult zebrafish (*Danio rerio*) gills. Groups of 8 individuals were exposed to a dose of each disinfectant corresponding to the therapeutic dose (TD) and five folds of the therapeutic dose (5xTD). Gills of all exposed zebrafish showed a higher occurrence of histopathological changes. These alterations included a slightly focal proliferation of interlamellar cells with obliteration of interlamellar spaces, mild infiammatory reaction with leucocyte infiltration and lifting of the epithelial layer from gill lamellae. Fish exposed to potassium permanganate showed more severe histopathological changes consisting of necrotic change of lamellar cells, distorsion and apical necrosis of secondary lamellae.

Introduction

The use of pharmaceutical substances is limited in fish, in contrast to mammalian therapeutics. It is basically restricted to anesthetic and anti-infective agents for parasitic and microbial diseases. Disinfectants are compounds that reduce pathogenic organisms on biological surfaces. Ideal characteristics of a disinfectant include a broad spectrum, fast action and low toxicity (Heit & Riviere, 1995). The most widely used disinfectants are iodophores, salts (potassium permanganate), organic chlorocompounds, aldheydes (formalin), hydrogen peroxide, quaternary ammonium compounds (benzalkonium chloride) and antiseptic dyes (malachite green) (Burka et al., 1997). In general, disinfectants are non-selective in their site of action, and lack selective toxicity. Furthermore, doses currently used to treat pet fish are, in most cases, based on empirical and anecdotal information, rather than on pharmacological and clinical data, with the result that fish toxicity may occur even at recommended dosages, particularly when fish of different species are treated at the same time (Stoskopf, 1988). Toxicity tests may be useful in order to evaluate the effects of xenobiotics at the organism level (Bernet et al., 1999) and histopathology could be used as an instrument or endpoint in toxicity studies on fish (Wester et al., 2002).

In this study we have investigated the gill toxicity of four disinfectants which are widely used in aquaculture, using zebrafish (*Danio*

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Group	Benzalkonium chloride		Formalin		Malachite green		Potassium permanganate	
	Dose (ppm)	Time of exposure	Dose (ppm)	Time of exposure	Dose (ppm)	Time of exposure	Dose (ppm)	Time of exposure
TD	2.0	1 h	250	1 h	2.0	0.5 h	5	1 h
5 x TD*	10.0	1 h	1.250	1 h	10.0	0.5 h	25	1 h

Table 1. Testing concentrations of the disinfectants employed in the test.
* TD = Therapeutic dose.

rerio) as test species. The four disinfectants examined in this study include: formalin (F), potassium permanganate (PM), benzalkonium chloride (BC) and malachite green (MG).

Materials and methods

Fish

Adult male zebrafish, 3.5±0.6 cm lenght, 0.60±0.2 g body-weight were purchased from ErreCI (Pisa, Italy). They were acclimated in 50 L glass aquaria containing dechlorinated tap water (temperature 23±1°C; pH 7.8) for at least 1 week before the experiment and fed on commercial pellets. Fish were kept under normal laboratory illumination with a 12h light/dark cycle. Procedures for the care and management of animals were performed in accordance with the provisions of the EC Council Directive 86/609 EEC, recognised and adopted by the Italian Government (DL 27.01.1992, n° 116).

Chemicals

F, BC, MG and PM were purchased (Sigma Aldrich, Milan, Italy).

Toxicity tests, exposure and mortality records

The tolerance of fish to each disinfectant was assessed after fish were exposed to the recommended therapeutic dose (TD) and five times the recommended dose (5xTD). The choice of each disinfectant TD and the duration of exposure were derived from the analysis of literature (Burka et al., 1997; Southgate, 1993). The exposure times were performed as follows: BC 1h, F 1h, MG 0.5h, PM 1h. Experimental groups also included untreated control fish, maintained in rearing water up to five folds of each disinfectant time exposure (BC 5h, F 5h, MG 2.5h, PM 5h) (table 1).

Fish were divided into nine groups with eight fish each (8 test groups and one untreated control) and kept in 20 L recirculating system aquaria. Then they were transferred into test aquarium (aerated to restore the concentration of dissolved oxygen to at least 90% of its air saturation value) where they were subjected to static bath exposure of each of the 4 disinfectants at the TD and 5xTD. A calculated volume of each disinfectant was added to each test aquarium based on its volume of water. Before addition, each disinfectant was diluted in 1 L of water removed from the test aquarium and then mixed throughout the test aquarium. The control group was exposed to untreated water. At the end of treatment, fish were then returned to untreated aquaria and monitored for up to 96 hours.

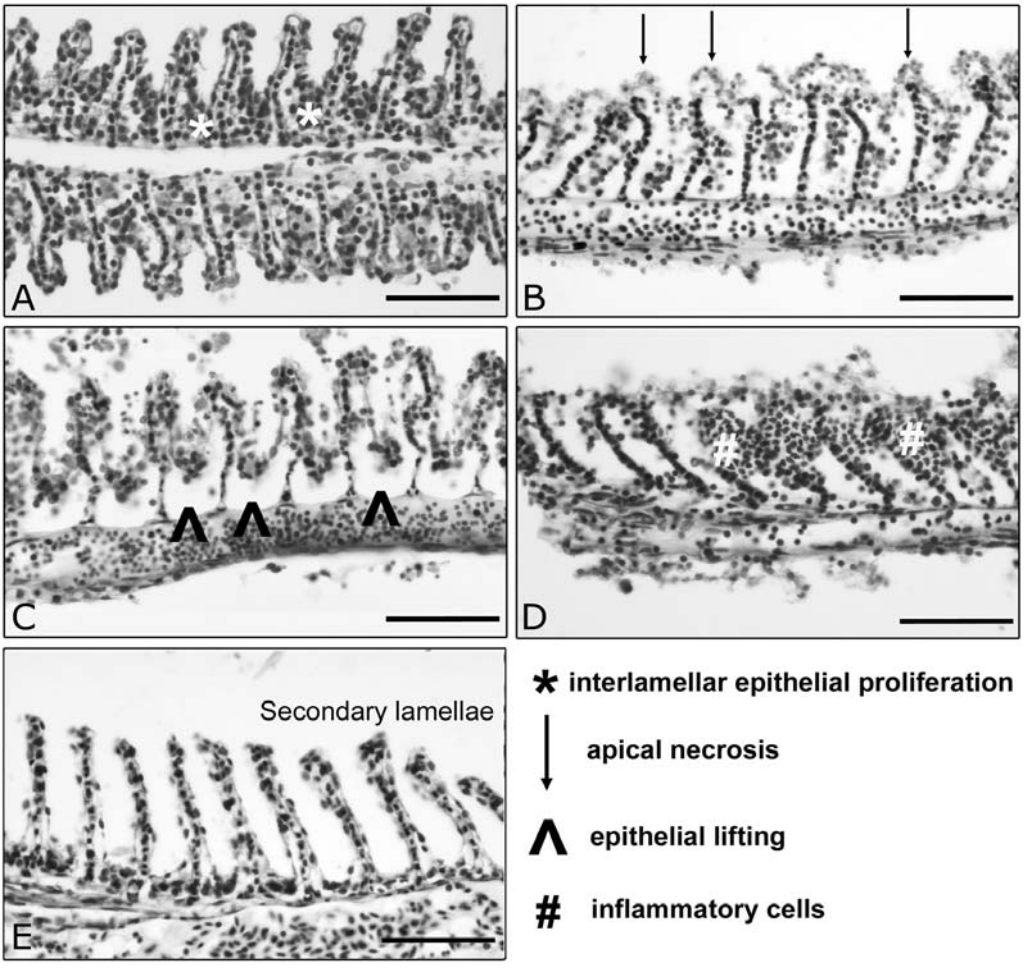

Figure 1. Gill histological sections of adult zebrafish; (A) Fish exposed to 1250 ppm F for 1 h. Note a slight proliferation of lamellar and interlamellar epithelium and epithelial lifting; (B) Fish exposed to 25 ppm PM for 1h. Severe degeneration and necrosis of the epithelial cells; (C) Fish exposed to 10 ppm MG for 0.5 h. Separation of secondary lamellar epithelium from the pillar cell (lamellar edema); (D) Fish exposed to 10 ppm BC for 1 h. Infiltration of inflammatory cells between the layers of the lamellar epithelium and epithelial lifting; (E) Normal gill structure from control zebrafish (H&E stain, scale bar = 50 μm).

Histopathological procedures and assessment

All exposed fish were collected immediately after death. The other fish were sacrificied at the end of the 96 hours. All fish were fixed in a 10% buffered formalin solution (pH 7.4) for 24 hours and routinely processed. Whole body para-sagittal sections were prepared. Gill morphological alterations were classified into one of these main categories: proliferative (increase of the number of specific cell types), degenerative (breakdown of tissue and/or cells), inflammatory (increased presence of cells in tissue repair; response to damaged

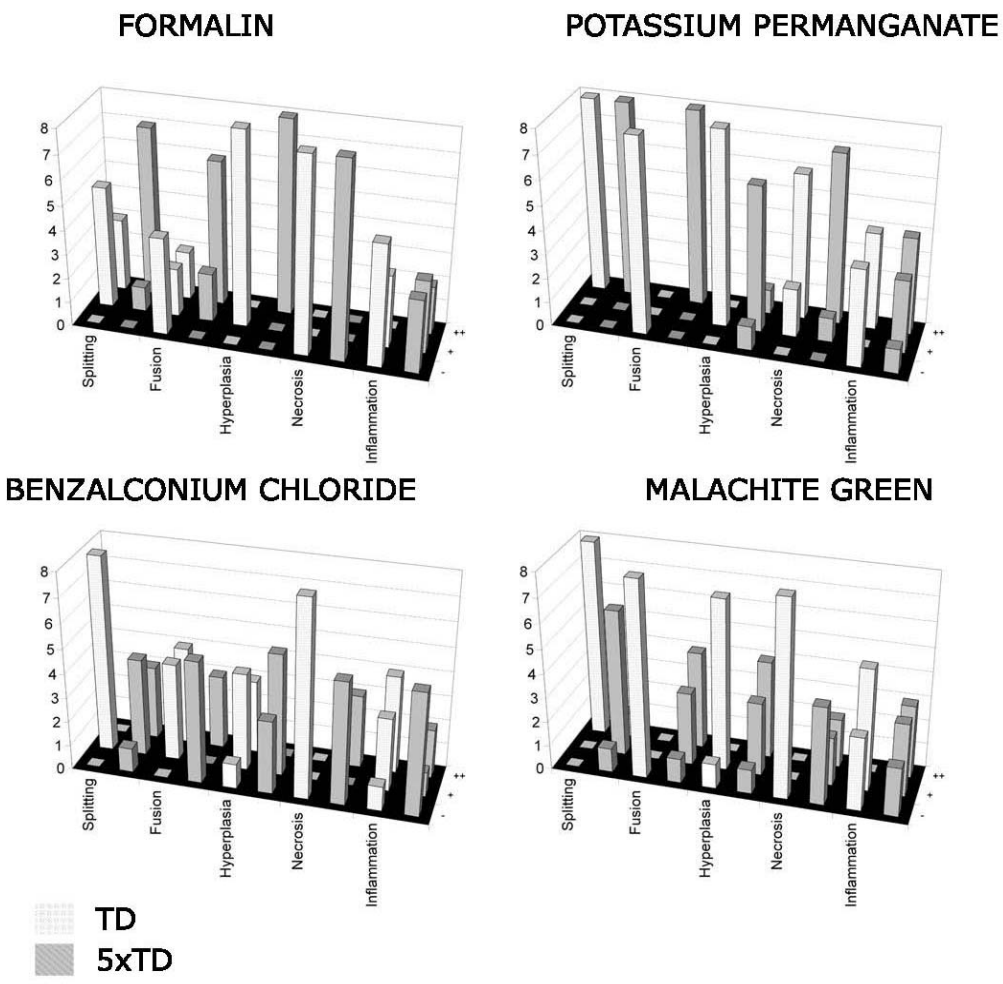

Figure 2. Semiquantitative evaluation (score assessment) of histopathological alterations in the gills of zebrafish exposed to each disinfectant, at TD and 5xTD.

tissue) and structural (changes in tissue architecture), each representing a general tissue response to a particular stressor.

The degree of histopathological alterations was qualitatively and semi-quantitatively evaluated for each individual fish, by assessing a score to tissue lesion severity and comparing it to control sections. Ranking was as follows: - = no pathological alterations; + = mild to moderate focal changes; ++ = severe pathological alterations. The cumulative effect of disinfectants exposure to histopathological gill alterations was then assessed.

Results

Histopathological cumulative findings

Controls

In histological sections obtained from all zebrafish from the control group each gill was made up of filaments of primary lamellae. Secondary gill lamellae, originating from the filaments in double rows, were disposed perpendicularly to each filament and were covered by a single layer of normotipic flattened respiratory epithelium (figure 1E).

F- exposed fish

Histopathological changes in the gills were characterized primarily by epithelial cell proliferation at the base of the secondary lamellae resulting in obliteration of the interlamellar space and, in some cases, in fusion of secondary lamellae. Therefore the major point of interest in F-exposed fish was the hyperplasia of the respiratory epithelium (figure 1A). Inflammatory alterations, such as lamellar edema, appeared as a separation of the respiratory epithelium from the pilar cells in the secondary lamellae.

PM-exposed fish

The predominant findings consisted of degenerative and necrotic changes of respiratory epithelium and pilar cells (figure 1B) and focal obliteration of the interlamellar spaces by an inflammatory exudate containing necrotic material. The majority of exposed fish showed numerous gill arch filaments that were disorganized and in most cases secondary lamellae exhibited distorsion, apical necrosis and fusion. Inflammatory alterations consisted mainly of a lifting of the gill epithelium (epithelial lifting), and an infiltration of leukocytes and lymphocytes in the secondary lamellae.

MG- and BC- exposed fish

The lesions observed in the gills of the MG- and BC- exposed animals exibithed similar histopathological features. Hyperplasia of interlamellar cells could be observed in a few animals. Multifocal degenerative and necrotic areas, which involved the epithelial cells both at the tip and along the edge of secondary lamellae, were also observed. The most common change, which was observed in almost all cases, was the lifting of the epithelium layer from gill lamellae due to lamellar oedema (figure 1C). Inflammatory infiltrate, which was represented by cells located in the region between the pilar cells and the outer layer of lamellar epithelium, was a finding in both groups (figure 1D).

The overall score assessment to tissue lesion severity for TD and 5xTD exposure is reported in Figure 2. Fish exposed to the 5xTD showed a higher level of histopathological alteration when compared to the TD exposed ones.

Discussion

Zebrafish, a cyprinid, is a relevant fish species for ornamental aquaculture and a model for toxicity tests (Spitsbergen & Kent, 2003). In the present study gill alterations following exposure to F, BC, PM and MG were histologically evaluated on test-zebrafish. The tolerance of F, PM, MG and BC exposure for zebrafish have been previously evaluated by Meucci et al., (2005). The authors graded the toxicity of disinfectants for zebrafish as MG < BC < F< PM.

The range of the structural alterations of the gill lamellae observed included lifting of lamellar epithelium, swelling and fusion of secondary lamellae, hyperplasia of lamellar

and interlamellar cells, necrosis and inflammation. All the categories of alterations were present, with different scores, in all dead and killed fish after disinfectant exposure both at the TD and 5xTD. The higher severity of histopathological alterations detected in the group of fish exposed to the 5xTD correlated well with the mortality records. Moreover, the presence of histopathological alterations in the TD exposed fish which did not show mortality in a 96 h period underline the relevance of histopathological assessment to detect sublethal alterations.

F-exposed fish showed predominantly proliferative alterations, while PM-exposed fish exhibited necrotic changes. Gill pathological features such as inflammatory alterations and hyperplasia of interlamellar epithelium were similar in MG and BC exposed fish.

The observed proliferative changes might represent a physiological adaptative response which reduces the vulnerable surface area of the gills rather than a direct effect of toxicants. In addition to this, hyperplasia of the lamellar epithelium, as well as epithelial lifting, increases the distance across which waterborne irritants must diffuse to reach the bloodstream. These changes may be advantageous for fish by reducing toxicant entry, but the efficiency of gas exchange will be severely impacted (Hemalatha & Banerjee, 1997). Despite the widespread use of F, fish farmers and fish health professionals are concerned about its potential to damage branchial architecture (Speare et al., 1997). Previous reports (Rucker, 1962) recorded that even a single F treatment of juvenile rainbow trout, *Onchorhynchus mykiss*, at recommended dose levels, was seriously damaging to the gill of some fish. Our study in zebrafish exposed to F demonstrates gill alterations, whether these are non-specific response pattern or directly related to the toxicant exposure is still to be investigated.

Necrotic changes were the predominant lesions observed in the PM exposed fish. These findings are in accordance with data reporting that PM was the least "safe" disinfectant for zebrafish (Meucci et al., 2005). Necrotic lesions are believed to reflect the direct deleterious effects of toxicant, they are generally irreversible, and their persistence and progression may lead to a partial or total loss of organ function (Nero et al., 2005). Since the permanganate ion MnO_4^{2-} is a strong oxidant (Tucker & Boyd, 1977), the mechanism of toxicity may be mediated through oxidative stress.

Different patterns of histopathological alterations, including cell necrosis, were detected in BC- and MG-groups, however these were less severe. Gill lesions have previously been described in healthy rainbow trout after BC-exposure at a dose minimally above recommended treatment levels (Byrne et al., 1989) and following repeated exposures to MG (Gerundo et al., 1991), but histological lesions have not been documented.

Inflammatory alterations, such as lamellar edema and inflammatory infiltrate, were found in almost all exposed fish and the lifting of the secondary lamellar epithelium likely contributes to changes in gill interlamellar distance. The amount of inflammatory infiltrate mainly represented by neutrophils was generally mild and focal. Infiltrating

leukocytes may release cytokines which in turn can activate mononuclear phagocytes and directly induce synthesis of the enzymes that mediate the respiratory burst.

In conclusion, our study demonstrates gill histopathological alterations in fish exposed to F, BC, PM and MG, with distinct response pattern identified in the F and PM groups and with different level of severity based on the dosage of the disinfectant used. Further investigations are needed to understand the role of each disinfectant in the pathogenesis of both specific and aspecific response patterns.

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