Original Article

Safety and Toxicity Evaluation of Bronopol in Striped Catfish (*Pangasianodon hypophthalmus*)

Patharapol Piamsomboon [1] Mintra Lukkana [2] Janenuj Wongtavatchai [3*]

Abstract

The study was performed to evaluate the safety and toxicity of bronopol in striped catfish *Pangasianodon hypophthalmus*. Bronopol product is licensed in Thailand and Vietnam for control of aquatic fungi and ciliated protozoa in scaleless fish at the daily dosage of 10 mg/l, 2-hour static immersion, for 5 consecutive days. Safe concentrations were evaluated in fish exposed to bronopol at the therapeutic dosage (10 mg/l, 2 hours, 5 consecutive days) and 2 and 3 times overdose (20 and 30 mg/l, 2 hours, 5 consecutive days). Fish treated with 10-30 mg/l bronopol for 2 hours daily for 5 consecutive days appeared clinically healthy and no adverse effects were found throughout the test period and for 30 days post treatment. These results show that the concentrations of bronopol of up to 3 times the therapeutic concentration are safe at 2-hour exposure. 24-hour median lethal concentrations tested in 3.5 g (SD ± 0.6) and 6.7 g (SD ± 1.2) fish were 11.04 and 11.18 mg/l, respectively, indicating that continuous immersion at the therapeutic dose can induce toxic effects. Therefore, bronopol is safe for use on striped catfish when it is administered at the therapeutic dosage.

Keywords: bronopol, LC_{50}, *Pangasianodon hypophthalmus*, toxicity

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บทคัดย่อ

ความปลอดภัยและความเป็นพิษของโบรโนพอลต่อปลาสวาย (*Pangasianodon hypophthalmus*)

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โบรโนพอลเป็นสารเคมีที่ประเทศไทยและประเทศเวียดนามใช้รักษาและควบคุมโรคที่เกิดจากรา น้ำและซิลิเอตโปรโตซัวในปลาไม่มีเกล็ดได้ตามกฎหมายโดยใช้แช่ปลาที่ความเข้มข้น 10 มิลลิกรัมต่อน้ำ 1 ลิตร เป็นเวลา 2 ชั่วโมง ติดต่อกัน 5 วัน การศึกษานี้เป็นการประเมินความปลอดภัยและความเป็นพิษของโบรโนพอลต่อปลาสวาย (*Pangasianodon hypophthalmus*) โดยทดสอบในขนาดการใช้ที่กำหนด (10 มก./ลิตร 2 ชั่วโมง ติดต่อกัน 5 วัน) และ 2 และ 3 เท่าของขนาดการใช้ที่กำหนด (20 และ 30 มก.ต่อลิตร เป็นเวลา 2 ชั่วโมงในน้ำนิ่ง ติดต่อกัน 5 วัน) การทดสอบพบว่าปลาสวายที่แช่ด้วยโบรโนพอลขนาด 10-30 มก.ต่อลิตร เป็นเวลา 2 ชั่วโมง ติดต่อกัน 5 วัน ไม่แสดงความผิดปกติตลอดระยะเวลาการทดลองและ 30 วันภายหลังสิ้นสุดการทดลอง แสดงว่าโบรโนพอลที่ความเข้มข้น 3 เท่าของขนาดที่กำหนดมีความปลอดภัยหากกำหนดเวลาแช่เพียง 2 ชั่วโมงอย่างไรก็ตาม median lethal concentration ที่ 24 ชั่วโมง (24 h LC50) ของโบรโนพอลในปลาสวายขนาด 3.5±0.6 กรัม และ 6.7±1.2 กรัม พบว่าเท่ากับ 11.04 และ 11.18 ตามลำดับ แสดงให้เห็นว่าการแช่ปลาในโบรโนพอลตามความเข้มข้นที่กำหนดแต่ระยะเวลาต่อเนื่องเกิน 2 ชั่วโมงเป็นอันตรายต่อปลา ดังนั้นการใช้โบรโนพอลตามขนาดและระยะเวลาที่กำหนด มีความปลอดภัยต่อปลาสวาย

คำสำคัญ: โบรโนพอล LC50 ปลาสวาย ความเป็นพิษ

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Introduction

Striped catfish, *Pangasianodon hypophthalmus*, a scaleless facultative air breather (Lefevre et al., 2011), is a major freshwater aquatic species cultured in South-east Asia. One billion tons per year of striped catfish are farmed in Vietnam, leading the world's white fish production and gaining export income of over $1.40 billion (FAO, 2011; Sliva and Phuong, 2011). Intensive culture system has produced stressful environments that cause an increase in susceptibility to opportunistic pathogens, including pathogenic bacteria, external parasites and aquatic fungi that are normally present in water sources of fish culture facilities (Gaikowski et al., 1999; Davis et al., 2003). Aquatic fungi in the family *Saprolegniaceae* and ciliated protozoa *Ichthyopthirius multifiliis* are common opportunistic pathogens that can cause a dramatic loss in freshwater fish production (Rach et al., 2004; Francis-Floyd and Reed, 2009). Conventional treatment for control of fungal or protozoa infection with a triarylmethane dye, malachite green, is highly toxic to the fish and the dye may pass through the food chain and cause carcinogenic, mutagenic and teratogenic effects in consumers (Wan et al., 2012). Malachite green is now banned for use in food fish in many countries, including Thailand. Formalin has been used as an alternative, but can only be used under strict control because of its irritant properties (Cruz and Pitago, 1989). Given this situation, less toxic compounds are needed for control of fungal or protozoa infection in fish.

Bronopol (2-bromo-2-nitro-1, 3-propanediol) is a broad-spectrum disinfectant that has a wide range of applications, including treatment and control of fungal infection and water disinfection in food production, pharmaceutical and cosmetic industries (Treasurer et al., 2005; Birkbeck et al., 2006). Bronopol is used for treatment of many aquatic animal diseases, including fungal, parasitic, bacteria and myxobacteria infection. The use of bronopol has become an alternative choice in aquaculture industry since malachite green was banned and formaldehyde was reclassified as carcinogen in humans (Picón-Camacho et al., 2011). The European Agency for Evaluation of Medicinal Products (EMEA) enrolls bronopol as a safe chemical for aquaculture and it has been used in European countries for salmonid egg disinfection and treatment and control of *Saprolegnia* infection (Grant, 2002; Srivastava et al., 2004). Pyceze® (Novartis Animal Health, Litlington, UK) is a well-known commercial bronopol product that is licensed in Europe for disinfection of salmonid eggs and reduction and control of fungal infections (*Saprolegnia* spp.) in farmed Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*.

Acute toxicity of bronopol has been tested in several fish species, including sheepshead minnow *Cyprinodon variegatus*, bluegill *Lepomis macrochirus*, and rainbow trout (US EPA, 1995; Madsen et al., 2001); however, bronopol toxicity in scaleless fish has not been documented. Toxicological assessment of the chemical to fish is mandated legally in establishment of a commercial bronopol product in

Thailand; therefore, the present study was performed to examine the safety of bronopol in striped catfish fingerlings. Toxicity of the compound was examined on the median lethal concentration (LC_{50}) in 24 hours exposure and histological changes in exposed fish.

Materials and Methods

Animals: The study was carried out in a striped catfish hatchery in Chachoengsao province, Thailand, during the months of September-October 2011. Fish weighing approximately 3.5 g (SD±0.6) and 6.7 g (SD± 1.2), age groups which are most likely to be treated with the ectoparasiticide compound, were used for the study. Five percent of the fish in both weight classes were randomly selected for a pre-study health examination (PSHE) and bacteriological analysis to ensure suitable health status of fish for the trial. Fish that passed the PSHE were stocked for 7 days acclimatization in a flow-through system and the stocking density was 500 fish/500 L-tank. Two stocking tanks were used to separate fish of each size; one for 3.5 g (SD±0.6) and the other for 6.7 g (SD±1.2) fish. The fish were given commercial dry pellet twice a day. During the study, water parameters in the experimental aquaria indicated a well-oxygenated environment (dissolved oxygen ranged from 8.1 to 8.5 mg/l), a temperature of 28-30^{0}C, pH 7.0-7.5, 0.4-0.6 mg/l ammonia and 0% salinity. The fish appeared clinically healthy and < 5% mortality was observed, which is suitable for testing of chemicals (OECD, 2013).

Chemical: A commercial bronopol product Antizol® (Virbac Asia Pacific Co, Ltd, Bangkok, Thailand) containing 50% bronopol w/v, was used in the study. The concentration of bronopol applied in the test was nominally determined based on the product certificate of analysis (COA), and a fresh solution of bronopol was prepared for each assay.

Safe Concentration: The 6.7 g (SD±1.2) striped catfish were used for safety evaluation. Fish from the holding tanks were impartially allocated to 12 of 20 l-flow through aquaria (10 fish/20 l aquarium). The assay was run in triplicates with a static exposure to 0, 10, 20, 30 mg/l bronopol for 2 hours, once a day for 5 consecutive days. Appropriate aeration was maintained during the test and the flow through system was resumed immediately after the assay. Clinical observations and mortality of the tested fish were made during the exposure and 30 days post-exposure period. At 30 days post-treatment, two fish in each group were collected using dipnetting for histological examination. Fish tissues that were subjected to the systemic toxicants; gills, liver and spleen, were fixed in 10% formalin for 24 hours. Specimens were sectioned at 4 µm and stained with Hematoxylin and Eosin (H&E) for histopathological evaluation.

Toxicity assay: Toxicity of bronopol was determined based on the 24-hour median lethal concentration (LC_{50}). Experiments were conducted on fish in both weight classes, the 3.5 g (SD±0.6) and 6.7 g (SD±1.2) fish. The fish were randomly assigned into triplicate of 7 groups (15 fish per group), each group maintained in a 30 L container. Three groups of fish were tested with each combination of fish weight and concentration at 0, 5, 10, 15, 20, 25 or 30 mg/l bronopol. Dead fish were removed every 1-2 hour for the post-mortem evaluation. At 24 hours, water in the immersion tank was completely replaced with stock water. Feeding was withheld 24 hours before and during the assay. Clinical signs and behavior changes of treated fish were observed every 1-2 hour during the experiment and for 30 days post-exposure. The accumulative mortality at 24 hours exposure was calculated for LC_{50} values using Probit analysis (SPSS version 17.0; IBM; USA). Twenty percent of survivors at 5 and 14 days post-exposure were processed for histological examination. Fish tissues; gills, liver and spleen, were prepared for histological examination.

Results

No adverse effect concentrations: Daily 2-hour immersion of bronopol at up to 30 mg/l for 5 consecutive days resulted in no adverse effect in the exposed fish. The fish demonstrated normal behavior during the treatment and for 30 days post-treatment. Histological examination of gills, liver and spleen sampled from fish at the end of the experiment showed no differences between the treated groups (10, 20, and 30 mg/l bronopol) and the untreated controls.

Lethal concentrations: The cumulative mortalities in striped catfish fingerlings for different exposure concentrations at 24 hours are shown in Fig 1. Bronopol at 20 mg/l caused high mortality in both weight classes within 12 hours post immersion. The 24-hour LC_{50}values of bronopol calculated with Probit analysis were11.04 mg/l (95% CI = 10.06-12.02) in 3.5 g (SD±0.6) fish and 11.18 mg/l (95% CI = 10.49-11.89)

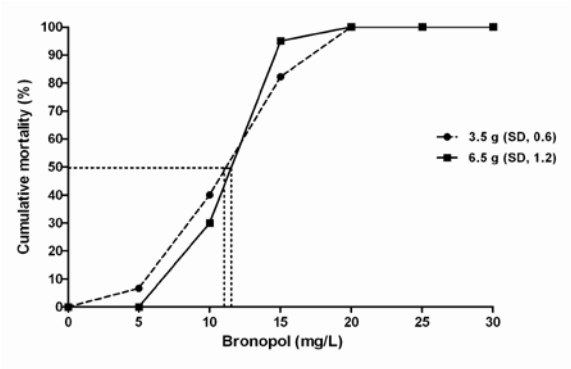

Figure 1 Toxicity assay; cumulative mortality of striped catfish were observed in 24-hour immersion with different concentrations of bronopol.

in 6.7 g (SD±1.2) fish. The 24-hour exposure showed lethal toxicity in all treated groups and even the subtherapeutic concentration of 5 mg/l bronopol caused 6.67% mortality.

Behavior changes: There was no behavior change in the groups exposed to bronopol for < 4 hours. A change in behavior was first observed at 6 hours in the groups treated with ≥10 mg/l bronopol and was more evident in the fish exposed to higher

concentrations. The nonlethal toxic effects of bronopol provoked behavioral changes in the exposed fish. The distressed fish expressed behavior indicating membrane irritation such as agitated swimming, problems with breathing, and hyperemic skin with excess mucus excretion.

Gross and histological findings: Severe hyperemia of opercular tissues was noted in all treatments of 24-hour exposure. Lesions reflecting increased toxicity were found with higher exposure concentrations and durations. Fresh gill tissue preparations showed extensive congestion of gill lamellae. Histological lesions indicated acute inflammation of gill tissues and thickening of primary and secondary lamellae with prominent infiltration of inflammatory cells, with complete fusion of the secondary gill lamellae found in severe cases (Fig 2). However, histological examination of hepatic and splenic tissues did not show significant changes related to exposure to the test compound. Splenic congestion and hydropic degeneration of hepatic cells were observed in all groups, including the control fish.

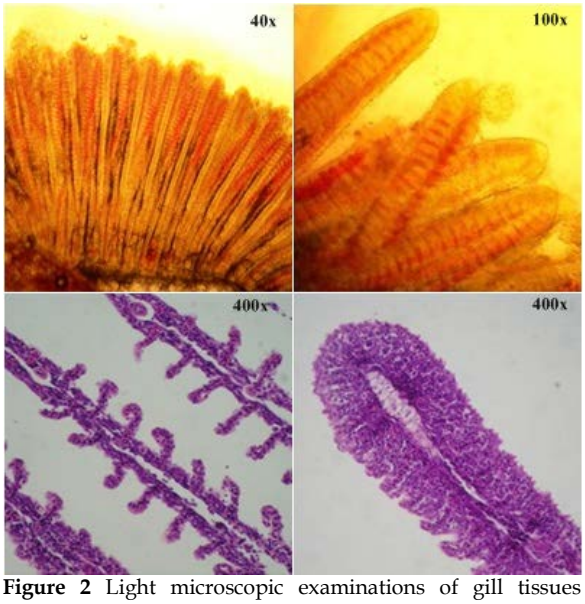

Figure 2 Light microscopic examinations of gill tissues obtained from fish exposed to 10 mg/l bronopol for 24 hours. Fresh tissue preparation showed acute hyperemic gill lamellae (upper panel). Histological lesions (H&E stain) showed acute inflammation of gill tissues (lower panel). Gill lamellae were infiltrated with inflammatory cells, causing complete fusion of the secondary lamellae in severe cases.

Discussion

In the present study, we evaluated the lethal dose of bronopol in striped catfish fingerlings to establish a proper treatment protocol and minimize toxic effects. The LC_{50} values of bronopol have been reported in several fish species. Toxicity tests in 5 g sheepshead minnow gave 24-hour and 96-hour LC_{50} values of 101 and 59 mg/l bronopol, respectively (US EPA, 1995). Madsen et al. (2001) reported a 96 hours LC_{50} value of 35.7 mg/l of bronopol in 1 g bluegill. The toxicity of bronopol observed in this study appears to be higher than that reported in previous studies on other species. The 24-hour LC_{50} value analyzed from the toxic response was approximately 11 mg/l bronopol for both weight classes, 3.5 g (SD±0.6) and 6.7 g (SD±1.2) striped catfish. Toxicity in an aquatic animal is affected by water temperature, pH, hardness, alkalinity, and species differences; and generally warm water fish are more sensitive to toxicants than coldwater fish (Gaikowskiet al., 1999). The increased toxicity of bronopol at higher temperature and pH might occur because these conditions accelerated the decomposition rate of bronopol to 2-bromo-2-nitroethanol (BNE) and bromonitromethane (BNM), which were more stable, but also more highly toxic compounds (Cui et al., 2011). The warm water habitat and scaleless physical nature of striped catfish may make them prone to bronopol toxicity and may account for the low LC value for bronopol compared to those found in other fish species.

The similar 24-hour LC_{50} of bronopol in striped catfish with different body weights found in this study suggests that 24-hour continuous exposure was beyond the tolerance level of the fish for bronopol. In addition, this value is close to the therapeutic concentration of 10 mg/l bronopol. Therefore, the dosage duration is critical, particularly since the extended duration clearly showed harmful effects to the fish, even at 5 mg/l bronopol. In contrast, concentrations up to 30 mg/l bronopol were safe with 2-hour exposure. The fish treated with 30 mg/l bronopol for 2 hours did not show clinical or histological adverse effects throughout the treatment period and in 30 days post-treatment.

Histologically, marked lesions were found in gill tissues. Deformity of gill lamellae increased with increased bronopol exposure and complete fusion of secondary lamellae occurred after prolonged exposure. The microscopic lesions of the gills of the fish revealing toxicity had similar characteristics to those in other reports of fish exposed to formalin (Reardon and Harrell, 1990), copper sulfate (Karan et al., 1998), and deltametrin (Yildirim et al., 2006). Subepithelial edema was also apparent in gill filaments in fish exposed to a toxic level of bronopol. Karan et al. (1998) suggested that subepithelial edema occurred frequently in fish in response to toxicants, since this physiological response tended to increase the diffusion distance of the toxicant to epithelial cells. In addition, edematous fluid provides physiological evidence of an inflammatory reaction to dilute toxic substances surrounding gill lamellae. The damaged gill lamellae may reflect circulatory dysfunction that

impairs their ability to regulate oxygen consumption, ammonia excretion and osmoregulation. Some fish survived the overdose exposure, but disorder of gill functions due to toxicant exposure may affect the fish health, making them less tolerant to environmental insults.

This study was performed to assess the safety of application of bronopol in striped catfish fingerlings. The therapeutic dosage of 10 mg/l bronopol administered for 2 hours for 5 consecutive days was proven to be safe for striped catfish weighing ≥ 3 g, and 2 hours static immersion of up to 30 mg/l bronopol for 5 consecutive days was not harmful to the fish. Although daily 2-hour immersion of bronopol is considered safe at up to 3 times the therapeutic concentration, the natural characteristics of striped catfish, which are scaleless fish that live in a warm water habitat, may make them susceptible to bronopol toxicity. The reported 24-hour LC_{50} of 11 mg/l is close to the therapeutic dose of 10 mg/l bronopol, and so it is important to note that administration of this compound should adhere to the specific dosage and exposure period.

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