Journal of Aquatic Animal Health
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uahh20

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Version of record first published: 09 Jan 2011.

To cite this article: Jan Lovy, David J. Speare & Glenda M. Wright (2007): Pathological Effects Caused by Chronic Treatment of Rainbow Trout with Indomethacin, Journal of Aquatic Animal Health, 19:2, 94-98

To link to this article: http://dx.doi.org/10.1577/H06-042.1

Journal of Aquatic Animal Health 19:94–98, 2007

DOI: 10.1577/H06-042.1

[Communication]

Pathological Effects Caused by Chronic Treatment of Rainbow Trout with Indomethacin

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Abstract.—The purpose of this study was to determine adequate dose ranges and to test for side effects associated with chronic treatment of fish with indomethacin. Rainbow trout *Oncorhynchus mykiss* were orally treated with indomethacin at various nominal concentrations: a negative control (0 mg/kg) and low (5 mg/kg), medium (10 mg/kg), and high doses (15 mg/kg) daily for 30 d. A dose-associated mortality was observed, as cumulative mortality was 3, 13, and 33%, respectively, in the three indomethacin dose groups. No lesions were observed grossly or with histopathology in the control and low-dose treatment groups. Gross lesions were observed in the medium- and high-dose groups, including skin ulcers, abdominal distension, and necrosis of abdominal wall muscle. Histopathology of fish in the medium- and high-dosage groups revealed severe granulomatous peritonitis in which a large number of foreign body type giant cells were present around proteinaceous and plant material. The inflammatory response spread from the peritoneum through the somatic muscle to the epidermis, causing lesions within all layers of the skin. A large number of bacteria were noted within the peritonitis, observed both intracellularly and in large aggregates extracellularly. Perforations occurred within the anterior intestine, and the thick muscularis layer was replaced with inflammatory tissue. The present investigation shows that chronic indomethacin treatment produces gastrointestinal side effects in rainbow trout similar to those seen in mammals.

Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) that suppresses production of prostaglandins through inhibition of cyclooxygenase (COX) action on arachidonic acid. The drug suppresses the action of both the inducible COX-1 and constitutive COX-2. Suppression of the former is thought to result in serious side effects such as increased intestinal permeability and inflammation, which can lead to intestinal perforations. Specific COX-2 inhibitors have been developed that decrease risk of gastrointestinal toxicity but can still result in toxicity to the lower intestinal tract of mammals (Thiefin and Beaugerie 2005).

Several fish diseases result in an inflammatory response that may be more detrimental to the host than curative. For example, microsporidial gill disease in salmonids (MGDS) is characterized by the rupture of spore-filled cysts within the gills that elicits a severe inflammatory response; neutrophils engulf spores but are unable to eliminate them. The pronounced neutrophil infiltration is associated with severe gill tissue damage and is often associated with thrombosis within the intricate vascular network (Lovy et al. 2007). Down-regulation of the inflammatory response may be an efficient method for controlling MGDS. Another example is proliferative gill disease (PGD), a serious disease in farmed channel catfish *Ictalurus punctatus* that is associated with gill inflammation and hyperplasia in response to a myxosporean parasite. Fish with PGD that were treated with indomethacin developed less severe lesions than did nontreated fish, as demonstrated by histopathology (Davis 1994).

It appears that NSAIDs cause the suppression of prostaglandins in fish by inhibiting the COX pathway, as they do in higher vertebrates. Mozambique tilapia *Oreochromis mossambicus* treated with acetylsalicylic acid were shown to exhibit reduced levels of prostaglandins in their plasma (Van Anholt et al. 2003), and thrombocyte aggregation in rainbow trout *Oncorhynchus mykiss* was inhibited by treatment with indomethacin (Hill et al. 1999), although the mechanism of inhibition is unknown. Activated fish macrophages express COX-2 and it has been reported that release of prostaglandins by fish macrophages modulate inflammatory responses (Fast et al. 2005). In a macrophage-like cell line of Atlantic salmon *Salmo salar* there is a 30-fold increase in the expression of the proinflammatory cytokine IL-1β upon incubation with lipopolysaccharides and prostaglandin E_2 (Fast et al. 2005). Indomethacin ablated all generation of prostaglandin E_2 in the fish macrophage cell line (Zou et al. 1999).

With evidence showing that indomethacin has

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Received September 29, 2006; accepted February 13, 2007
Published online May 31, 2007

similar inhibitory action on prostaglandin generation in fish and mammals, applying the drug to fish that exhibit inflammatory diseases might be effective. Therapeutics for fish in aquaculture may be applied in various forms, but the most efficient method for salmonids in net-pens is probably through the feed. The intent of this study is to determine per os doses that are acceptable for rainbow trout and whether if side effects of the drug are present after chronic treatment at various doses.

Methods

Experimental design.—Rainbow trout were acquired from the Cardigan Fish Hatchery on Prince Edward Island and maintained at 10°C until 1 week before the experiment. Fish were approximately 6 months old and weighed about 18.5 g each. The fish were separated into four 70-L circular tanks maintained at approximately 15°C, with 30 fish per tank. The biomass of each tank was approximately 560 g ± 15 g. After allowing a 1-week period for fish to acclimate to the new tanks, they were treated with indomethacin (Novopharm; Toronto, Ontario) at various nominal concentrations, of which one group served as an untreated control. The treatment concentrations were applied at a control, low, medium, and high dose, which were at 0 , 5, 10, and 15 mg/kg of fish, respectively. The fish were given the appropriate nominal concentrations daily for 30 d.

In feed treatment.—Three-millimeter-diameter extruded pellets (Corey Aquafeeds; Fredericton, New Brunswick) were coated with indomethacin that had been mixed into gelatin (Oxoid; Basingstoke, UK). For every kilogram of feed to coat, 4 g of gelatin was dissolved in 70 mL of boiled distilled water and allowed to cool to about 25°C. Appropriate amounts of indomethacin were slowly added to the cooled gelatin mixture with constant mixing until the indomethacin appeared evenly dispersed throughout the mixture. The mixture of gelatin with indomethacin was slowly poured onto the feed with constant mixing and using a rubber policeman to evenly coat the feed. Feed was immediately spread over a tray to dry at room temperature for 1 h and then stored at 4°C until use. Appropriate feed treatment was given to the fish groups by feeding fish their respective treated feed at 2% body weight per day. We assumed a 2% weight gain per day and adjusted the feeding regime weekly. Mortalities were monitored throughout the treatment duration and amounts of feed given to groups were adjusted based on loss of biomass.

Sampling procedure.—When the experiment was ended (after 30 d), the remaining fish were sampled. All fish were examined grossly for external lesions, and five fish from each group were fixed in 10% neutral buffered formalin for histopathological examination. Incisions were made in abdomens of fish to allow better fixative penetration, and five fish from each group were transferred to jars containing 1 L of fixative. After routine processing, the tissue was embedded in paraffin wax, and 5-μm sections were cut, stained with hematoxylin and eosin, and examined with light microscopy. Two transverse sections were cut from each of the fish in all treatment groups, one section from the anterior portion of the body cavity through the liver and one from the area of the stomach and pyloric caeca.

Results

Feed palatability was good; fish continued feeding on the treated feed throughout the study, and no feed remained in any of the tanks. Mortalities in this study before termination of the experiment appeared to be treatment associated, such that the low-, medium-, and high-dose groups experienced 3, 13, and 33% cumulative mortality, respectively (Figure 1). Most mortalities occurred after 12 d of treatment; before death, those fish appeared inactive and were localized near the sides of the tank near the water surface.

Gross Lesions

Fish from the control and low-dose treatment groups had no external lesions. Fish from the medium- and high-treatment groups had large, irregularly shaped ulcers posterior to the pectoral fin on the lateral body surface. The central areas of the large ulcers were red with black margins, and the area on the skin peripheral to the lesion was pale (Figure 2A). We also observed circular skin ulcers located on the ventral abdominal surface that were variably dark red and black (Figure 2B). External lesions were observed in 30% and 25% of fish in the medium- and high-dose groups, respectively. Fish that had external lesions also had abdominal distension; the stomach was swollen and full of fluid, and abdominal muscle was excessively soft. There was no marked difference in severity of the lesions between the medium- and high-dose treatment groups.

Histopathology

No lesions were observed in any fish within the control group or the low-dose group. Fish from the medium- and high-dose group had several pathological alterations. A common finding was the presence of granulomatous peritonitis, which caused adhesions among the internal organs. Adhesions were observed between liver, gastrointestinal (GI) tract, spleen, and pancreas and frequently extended to the body wall

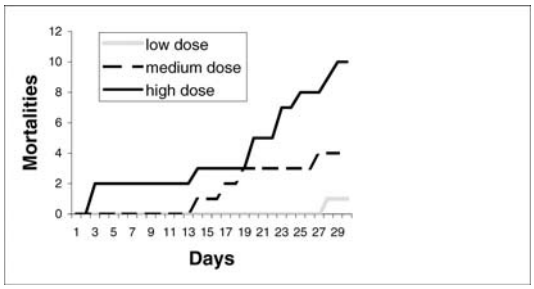

FIGURE 1.—Mortalities of rainbow trout given low (5-mg/kg), medium (10-mg/kg), and high (15-mg/kg) doses of indomethacin.

(Figure 3A). Abundant eosinophilic material, probably protein from feed contents, was found throughout the inflammatory infiltrate (Figure 3A). Many macrophages and fibroblasts were present in the lesion, and a consistent finding was a large number of foreign body–type giant cells seen within the infiltrate. The giant cells often contained eosinophilic material and in some cases cellular material containing cell walls, presumably plant material. The inflammation extended from the peritoneal cavity through the somatic muscle of the body wall (Figure 3B). The muscle and adipose tissue had degenerative areas, where the tissue was replaced with inflammatory cells (Figure 3B). The inflammation extended from the somatic muscle through the dermis and into the epidermis. This inflammation was associated with a loss of the adipose tissue of the hypodermis, loss of the adjacent iridophore layer, disorganization of the stratum compactum of the dermis, loss of scales and scale pockets in affected areas, and thickening of the epidermis accompanied by a reduction of mucous cells (compare the normal skin in Figure 3C with an affected area in Figure 3D). Another finding within the peritonitis was the presence of bacteria. The bacteria were extracellular within the inflammation, where large aggregates were formed, and intracellular, where cells were engorged with the bacteria (Figure 3E). Fish with peritoneal granulomatous inflammation also had lesions within the GI tract. In particular, areas of necrosis and severe inflammation were evident in the muscular tunic of the anterior intestine. The damage extended into the submucosa, where inflammatory cells covered an ulcerated area (Figure 3F).

Discussion

The work herein shows a detrimental effect to fish chronically fed medium and high concentrations of indomethacin. Mortalities occurred in all the treated groups in a dose-responsive manner, and no mortalities occurred in the control group. Mortalities were most prevalent in the high-dose treatment group (33%), followed by the medium-dose group (13%), and lastly the low-dose group (3%). An important finding is that fish treated over the study period with 10 and 15 mg/kg daily displayed severe peritonitis and ulcers in the anterior intestine. The abundant proteinaceous and plant material seen within the peritonitis most likely originated from the feed; probably an ulcer within the GI tract perforated and the feed within leaked out into the peritoneal cavity. The bacteria inhabiting the peritoneum may also have originated from the GI tract as a result of the perforation. The inflammation and bacteria probably damaged the muscle of the body wall, leading to a transmural ulcer extending from the peritoneum to the epidermis. This is consistent with the external finding of necrosis in the muscle of the peritoneal cavity and the gross skin lesions that were described. Granulomatous peritoneal inflammation frequently occurs in Atlantic salmon when given vaccines administered through the peritoneum. The inflammatory response occurs around the oil adjuvant and characteristically consists of macrophages, foreign body–type giant cells, and proliferating fibroblasts

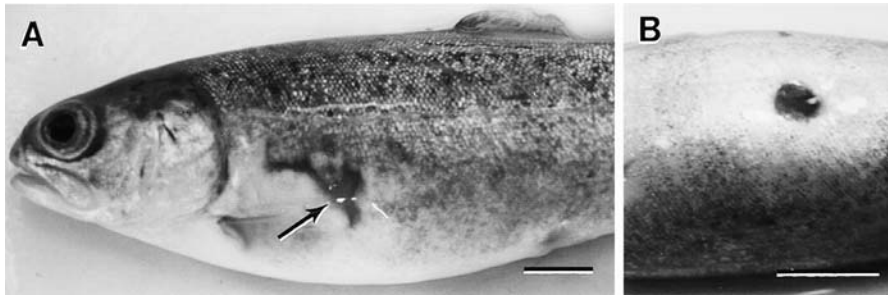

FIGURE 2.—Rainbow trout with skin ulcers after daily treatment with a medium (10-mg/kg) dose of indomethacin for 30 d. Panel (**A**) shows a large, irregular ulceration (arrow) located laterally, panel (**B**) a circular ulcer located in the ventral abdominal area; bar = 1 cm.

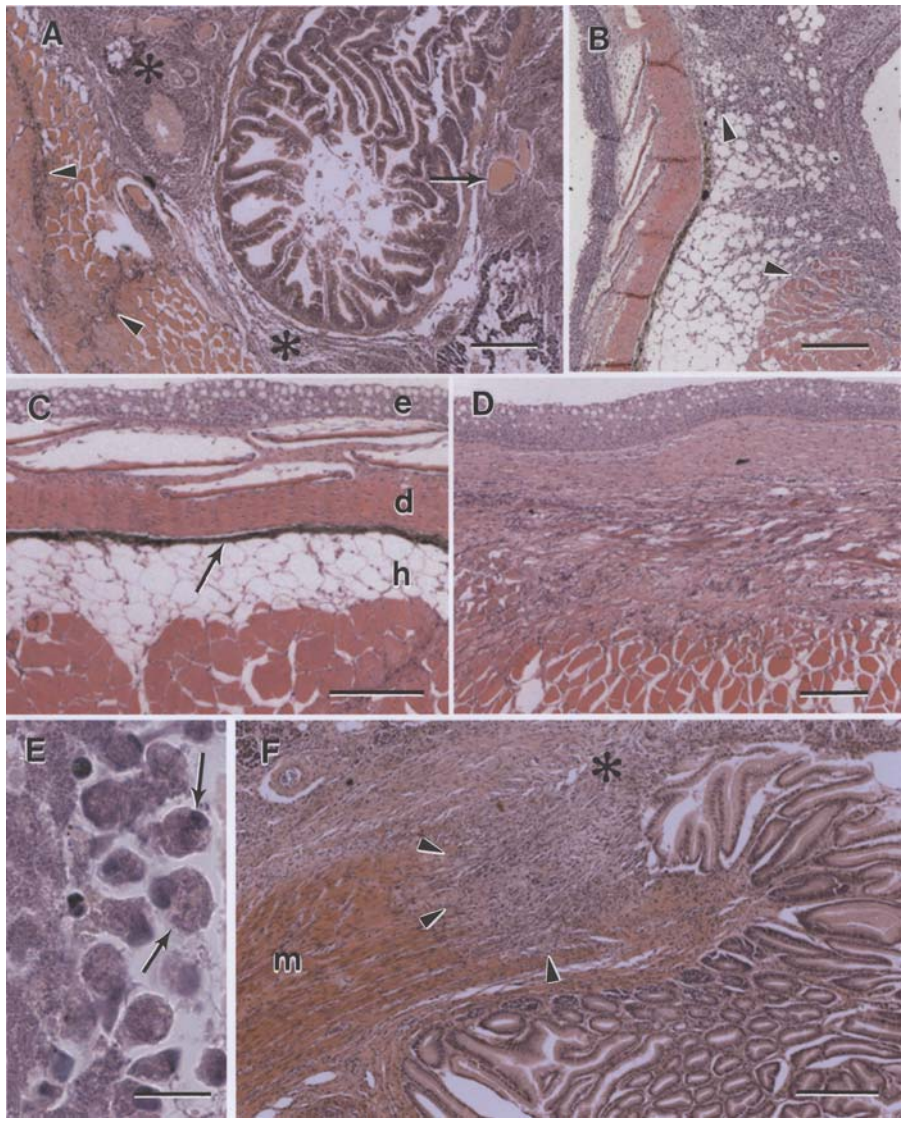

FIGURE 3.—Light micrographs of lesions associated with chronic treatment with medium (10-mg/kg) and high (15-mg/kg) doses of indomethacin. Panel (**A**) shows granulomatous peritonitis (asterisks) causing adhesion of the pancreas and gastrointestinal tract to the body wall. The inflammation extends to the muscle (arrowheads) of the body wall. The peritonitis contains eosinophilic material (arrow). Panel (**B**) shows a ventral body wall with inflammation originating in the peritoneal cavity (right) and extending to the skin (left). Inflammation is seen throughout the adipose and muscle tissue (arrowheads). Panel (**C**) shows an area of normal skin in which the epidermis (e), dermis (d), iridophore layer (arrow), and hypodermis (h) are illustrated. Panel (**D**) shows a skin lesion with inflammation extending from the muscle to the epidermis. Note the loss of the iridophore layer and hypodermis and that the dermal collagen layers have lost their densely packed arrangement. Panel (**E**) shows bacteria-engorged cells within the peritoneal cavity (arrows) and aggregates of extracellular bacteria (left). Panel (**F**) shows inflammatory tissue (asterisk) replacing the muscularis (m) and submucosa of the anterior intestine. Arrowheads show the boundaries of the inflammation and the intact muscularis layer. Bars = 250 μm in panels (A)–(D) and (F), 15 μm in panel (E).

(Ferguson 2006). This reaction is similar to the peritonitis seen in this study.

The toxic side effects of indomethacin have been thoroughly investigated for mammals, and damage to the GI tract is widely recognized (Brodie et al. 1970; Yamada et al. 1993; Evans and Whittle 2003; Thiefin and Beaugerie 2005). It is reported that every year 100,000 humans worldwide that use NSAIDs are

treated for adverse GI effects and 15% of them die of their complications (Bergh and Budsberg 2005). Small animals such as cats and dogs are also thought to be more sensitive to the consequences of NSAIDs than are humans (Bergh and Budsberg 2005). The early pathogenesis of gastrointestinal toxicity is reported to involve an increase in intestinal permeability, where the mucosa becomes vulnerable to injury from bile salts and intraintestinal bacteria. The bacterial injury leads to inflammation and intestinal ulcers, which can contribute to perforation and bleeding (Evans and Whittle 2003; Thiefin and Beaugerie 2005). Bacterial translocation, where bacteria cross the intestinal membrane into extraintestinal sites, can occur and it has been demonstrated that treatment with antibacterials during treatment with indomethacin leads to less severe intestinal injury and reduced bacterial translocation (Evans and Whittle 2003). Because this is the first study to examine the toxic side effects of indomethacin in fish, the pathogenesis of these effects is unknown. The intestinal perforation and inflammation in the muscularis of the anterior intestine seen in this study with rainbow trout is consistent with the indomethacin-induced lesions of mammals. Bacterial translocation from the GI tract to the peritoneum was also seen in the present study.

Interestingly, we observed GI toxicity in rainbow trout treated with indomethacin that was similar to what has been described for mammals. Because of variable feeding responses among fish within a group, some fish could easily receive higher doses than others; therefore, injection may be a better treatment method if precise dosing is required. According to this study, chronic per os treatment of indomethacin at 10 mg/kg or more daily results in severe toxicity. However, these doses may be tolerated with treatments of short duration; the first mortality in the 10 mg/kg treatment group was observed 12 d after the start of treatment. It appears that 5-mg/kg daily treatments are tolerated for prolonged periods, as only one mortality occurred in this group after 27 d of treatment. This study suggests that if indomethacin will be utilized as a therapeutic for inflammatory diseases in fish, then precautions similar to those used for mammalian treatment regimes must be taken.

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