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THE EFFECT OF OCHRATOXIN ON ANTIMICROBIAL POLYPEPTIDE EXPRESSION IN CHANNEL CATFISH (Ictalurus punctatus)

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ABSTRACT

Mycotoxins contamination of agricultural commodities poses a serious risk to animal health, including aquaculture species. While chronic effects of mycotoxins, including immunosupression, are much more important than acute toxicities, they are much less evident. Ochratoxin A (OA) is the most immunotoxic ochratoxin, yet little is known about its effect on immune function in fish. Antimicrobial polypeptides (AMPPs) are one of the most potent, innate, host defense factors, yet very little is known about what types of chronic stressors affect their expression. Among the most prevalent and potent AMPPs in fish are histone-like proteins (HLP). In this study, fish were fed 2, 4, or 8 ppm OA. Skin antibacterial activity was measured on Days 0, 28 and 56. Feeding 2, 4 or 8 ppm OA resulted in no changes in AMPP expression in any treatment group. Our data suggests that the immunosupp-ression of OA is probably due to impaired cell-mediated immune mechanisms other than a direct effect on antimicrobial polypeptide expression.

INTRODUCTION

Mycotoxins are secondary fungal metabolites which contaminate various types of feed commodities such as corn, wheat, cottonseed meal, peanuts, and soybean meal (Tuan et al 2003). Ochratoxin A (OA), produced by *Penicillium* and *Aspergillus* species, is one of the most important mycotoxins and causes major agricultural losses (O'Brien & Dietrich 2005). OA mediates its toxic effect by acting on cellular respiratory enzymes through competitive inhibition of ATPase, succinate dehydrogenase, and cytochrome C oxidase in mitochondria. Moreover, as a result of its phenylalanine moiety, it competitively inhibits phenylalonyl-tRNA synthase, thus disrupting protein synthesis. In addition, cellular damage is caused by hydroxyl radical formation and lipid peroxidation (Hussein & Brasel., 2001).

Ochratoxin A is toxic to fish, but susceptibility varies considerably among species. Sea bass (***Dicentrarchus labrax*** L.), are highly sensitive, having a 96 h LC50 of 9.23 mg OA/kg diet (El-Sayed et al., 2009). The LD50 for rainbow trout

(***Oncorhynchus mykiss***) by injection is 4.76 mg OA/kg (Doster et al., 1972). Channel catfish are much more resistant, tolerating as high as 4 mg OA/kg diet for at least 8 weeks without mortalities, and 8 mg OA/kg diet with 80% survival (Manning et al., 2003).

One of the most prevalent innate defenses in animals are antimicrobial polypeptides (AMPPs) (Zasloff 2002). Studies that have shown that depressed AMPP levels can greatly increase disease susceptibility. For example, recurrent bacterial infections occur in cases where there is a deficiency of α-defensins in neutrophils. Morbus Kostmann syndrome, which is a severe congenital neutropenia in humans that is typified by low concentrations of AMPPs in the mouth, results in recurrent oral infections (Pũtsep et al., 2002). Depressed levels of histatins, AMPPs in human oral mucosa, have been associated with an increased risk of developing human immunodeficiency virus (HIV) infection (Lal et al., 1992).

Among the most common AMPPs in fish are histone-like proteins (HLPs), which have high homology, to core nuclear histones. Histone-like proteins have been identified in skin, gill and/or spleen of channel catfish (***Ictalurus punctatus***) (Robinette et al., 1998), rainbow trout (Noga et al 2001, Noga et al 2002) and hybrid striped bass (striped bass ***Morone saxatilis*** ♂ x white bass ***M. chrysops*** ♀) (Noga et al., 2001, Noga et al., 2002). HLP-1 is the most prevalent and potent HLP, with broad-spectrum activity against bacteria, parasites and water molds (Robinette et al., 1998, Noga et al., 2002). HLPs are also the predominant AMPP in normal healthy channel catfish (Robinette et al 1998).

There have been no studies that have examined the effect of OA or any other mycotoxin on the expression of AMPPs in any animal. Thus, to better understand possible mechanisms underlying OA immunotoxicity, we assessed the effect of OA on the expression of AMPPs in channel catfish.

MATERIALS AND METHODS

Experimental Fish

Channel catfish (Ictalurus Punctatus) were obtained from a local producer and transported to North Carolina State University. Fish were acclimated in a 380 liter fiberglass aquarium at 24^{o}C for 60 days prior to beginning the experiment. During that time, fish were fed ad lib with a 2 mm pellet (40% crude protein, 10% crude fat, and 4% crude fiber, Zeigler Bros, Inc., Gardners, PA). Ammonia, nitrite, and pH were monitored weekly. Two weeks before initiating the experiment, fish were sedated with 60 mg/L buffered tricane and transferred to the experimental aquaria.

Experimental Design

Fifteen fish were placed in each of twelve, 60-liter freshwater aquaria (total N = 180). All aquaria were connected to a central filtration system having a conditioned biofilter (biocubes and bead filter [Aquadyne, Koi Camp Aquariology, Loganville, GA]) and a titanium heater (Process Technology Co., Mentor, OH). Fish began feeding normally almost immediately and were fed close to apparent satiation twice daily. After 7 days, the temperature

was increased from 24°C to 29°C over 7 days. After 14 d at 29°C (day 0), all fish were weighed and the day 0 sampling was performed. At day 1, all fish were switched to semi-purified diets (Table 1) prepared as described previously (Manning et al 2003), and having one of four concentrations of ochratoxin A (0, 2.0, 4.0, or 8.0 mg OA/kg feed). Triplicate aquaria were assigned to one of four treatments. During the experiment, 75% water changes were performed thrice weekly, and water quality was measured (ammonia, nitrite, nitrate, pH, and dissolved oxygen) twice weekly via water quality test kits (Aquarium Pharmaceuticals, Inc.).

Sample Collection

Three fish from each aquarium were sampled at days 0, 28 and 56. Each aquarium was sampled one at a time; the three fish were sedated with a low dose of buffered tricane (30 mg/L tricane + 60 mg/L sodium bicarbonate) and each fish was then euthanized one at a time in a separate container having a high dose of buffered tricane (200 mg/L tricane + 400 mg/L sodium bicarbonate). Each fish was weighed and measured to calculate the condition factor, K ($K = W \times 10^2 / L3$; W = weight in grams, L = total body length in centimeters). A skin scraping and a gill clip were then taken and examined immediately for pathogens under a light microscope. A skin sample was then collected for AMPP levels. Skin was processed for measuring AMPPs as previously described (Noga et al 2001, Robinette & Noga 2001). Briefly, 50 µl of tissue was collected to yield a total volume of 200 µl in 150 µl of 1% acetic acid (1:4 dilution of tissue). After boiling for 5 min, the sample was homogenized and then centrifuged at 14,000 x g for 10 min at 4°C. The resulting extract (supernatant) was used to measure antibacterial activity and HLP-1.

Bacterial Culture

Blood from representative fish in each treatment (2 fish per aquarium) was collected from the caudal vasculature with a 1 cc syringe having a 23 GA needle. A drop was placed on a Columbia blood agar plate and then spread with a sterile swab (Mini-tip Culturette, Becton Dickinson, Franklin Lakes, NJ). Culture plates were incubated at room temperature and observed daily for 14 d.

Total Antibacterial Activity

Total antibacterial activity was measured using the radial diffusion assay as described previously (Noga et al., 2002). Briefly, ***Escherichi coli*** D31 was propagated overnight in trypticase soy broth with 1% NaCl and then washed in PBS (pH 7) and diluted until to an OD_{570} of 0.10 with a Vitek colorimeter (Hach, Loveland, CO). One ml of the bacterial suspension and 1 ml of streptomycin sulfate (10 mg/ml stock) was added to an autoclaved agarose medium (1.57 g of low electroendosmosis agarose, 0.5 g of NaCl, 20.7 ml of 5X Luria-Bertani broth, 20.7 ml of 1 M phosphate buffer pH 6.7, and 58.6 ml of deionized water) that was cooled in a water bath to 45-48°C. Ten milliliters of the suspension was poured into each sterile Petri dish, which was used immediately or stored at 4°C until use. Samples were pipetted into 2.5 mm diameter wells punched in the agarose plate. After diffusion of the sample into the agarose, the plate was incubated

at 37°C for 18 h, at which time clearing zone diameters were measured by calipers to the nearest 0.1 mm. The radial diffusion assay clearing zone diameters were then converted to Units of activity by reference to a standard curve prepared by serially diluting calf histone H2B (Roche Diagnostics Co, Basel, Switzerland). The 1000 µg/ml concentration of calf histone H2B was considered to be 100 Units of activity. All values were multiplied by 40 to report the tissue concentrations (as µg/ml equivalents of calf histone H2B).

Statistical Analyses

All data were analyzed using SPSS version 17.0 (SPSS Inc, Chicago, Illinois). The effect of experimental variables (OA feed rate, aquarium replicate, sampling day) was tested by analysis of variance (ANOVA). A significant difference was accepted if $p \leq 0.05$.

RESULTS

Necropsy Examinations

There were no gross external lesion in any fish during the entire experiment; also, no pathogens were detected in skin or gill biopsies at any time. Blood cultures had a small number of bacterial colonies in some fish of each of the four treatment groups (ranging from 1 to 26 colonies). No predominant colony type was observed and in the sample with the largest number of colonies, three colony types were present.

AMPP Levels

There were no differences in the total antibacterial activity among any treatment groups at any of the sampling times (Fig. 1). This was also reflected in the lack of obvious changes measured in pooled extracts when measuring HLP-1 levels (Table 2). There was no detectable activity in these same samples against *E .ictaluri* (data not shown).

Condition factor (K)

Changes in condition factor (K) were less consistent; the 8 ppm treatment was significantly lower than the control at day 28, but was not different at day 56 (Fig. 2b).

DISCUSSION

Ochratoxin A is well-known to be immunosuppressive to vertebrates. OA reduces the size of immune organs, such as thymus, spleen, and lymph nodes, and the number of immune cells within tissues. It also causes profound alterations in the function of mononuclear leukocytes (Al-Anati & Petzinger, 2006), affecting production of reactive oxygen species, chemotaxis, phagocytosis and cytokine synthesis (Alvarez-Erviti et al., 2005).

Feeding 4 mg OA/kg feed for 10 weeks caused lymphoid depletion in the thymus of turkeys and was accompanied by reduced T-lymphocyte-mediated delayed cutaneous hypersensitivity responses (Dwivedi & Burns, 1985). In other studies, ochratoxin A inhibited natural killer (NK) cell activity and increased the growth of tumor cells in mice. Ochratoxin had little direct effect on NK cells, but did impair the induction of interferon. Because interferon is necessary for NK cell activity, the

suppression of NK cell activity was attributed to a decrease of endogenous interferon levels in the ochratoxin-treated mice (Luster et al., 1987). The effect of 0.5 or 2.0 mg OA/kg feed on broiler chicks revealed significant reductions in cell-mediated immunity, as indicated by diminished skin sensitivity, graft versus host reactions and T-lymphocyte counts. Total lymphocyte counts, total serum protein, serum albumin and serum globulin levels were significantly depressed on day 21. Phagocytosis was inhibited, and the number of splenic macrophages was drastically reduced in both intoxicated groups, as well as the weights of the thymus, bursa of Fabricius and spleen (Singh et al., 1990).

Holmberg (1988) showed a suppression of IL-2 production and phytohemaglutinin (PHA)-stimulated lymphoblastogenesis in porcine lymphocytes, suggesting that OA exposure results in a depression of cell-mediated immunity without involvement of the humoral immune system. A lack of overt effects on humoral immunity in pigs led to the suggestion that the immune-modulating effects of OA result from a suppression of lymphocyte blastogenesis and IL-2 production (Holmberg, 1988 and Harvey., 1992).

To our knowledge, we are the first to examine the effect of any mycotoxin on expression of any AMPP. However, Fujiwara et al. (2004) recently reported that 2-amino-1-methyl-6 phenylimidazo[4,5-b]pyridine (PhIP), a one of the most abundant heterocyclic amines produced while cooking meat and fish, induces an overexpression of α-defensin genes by Paneth cells in rats developing colon cancers.

Thus, our data did not show any effect of OA on antimicrobial polypeptide expression. Total antibacterial activity was not significantly different among treatment groups. These data suggest that OA does not affect AMPP levels in channel catfish.

Manning et al., (2003) found that in channel catfish fed OA for 8 weeks, survival was high for fish fed diets containing 0–4 mg OA/kg feed, but fish fed the diet containing 8.0 mg OA/kg had significantly lower survival. At 8 weeks, histopathological examination showed a significant increase in the severity of liver and posterior kidney lesions among catfish fed diets containing 2.0 mg OA/kg or above. These lesions were characterized as enlarged melanomacrophage centers which replaced normal hepatopancreatic or posterior kidney cells. Exocrine pancreatic cells, that normally surround the hepatic portal veins, were decreased or absent in fish fed 1.0 mg OA/kg diet or greater. Hematocrit was significantly lower for catfish fed 8 mg OA/kg of feed, but there were no significant differences in white blood cell (WBC) counts at any dietary levels of OA.

The condition factor (K) of the 8 ppm treatment was significantly lower than the control at day 28, but since the (K) was not different at day 56 and that of the control fish was similar to what has been reported for channel catfish (Perkins et al., 1997). Thus, condition factor changes were less consistent and did not reflect the lower innate immune status of the fish group fed the highest tested dose 8 mg OA/kg.

In summary, channel catfish exposed to moderate to high dietary levels of ochratoxin A (2-8 ppm) did not appear to be associated with depressed AMPP levels, suggesting that other aspects of impaired immune function are associated with ochratoxicosis.

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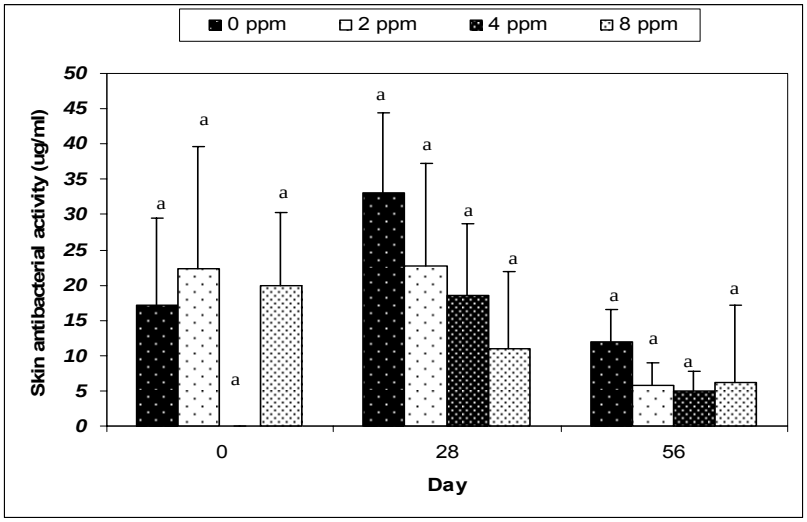
Table 1. Ingredient composition of the semipurified basal diet[a].

Ingredient	Amount (g/kg dry mixture)
Casein, vitamin free (USB #12866)[b]	290.0 g
Gelatin	80.0 g
Dexrin (USB #9004-53-9)	360.0 g
Cellulose	106.0g
Fish meal, menhaden	20.0 g
Carboxymethyl cellulose	30.0 g
Corn oil	30.0 g
Menhaden oil (USB #8002-50-4)	30.0 g
Mineral premix[c]	40 g
Vitamin premix[d]	12.5 g
Vitamin C[e]	1.5 g

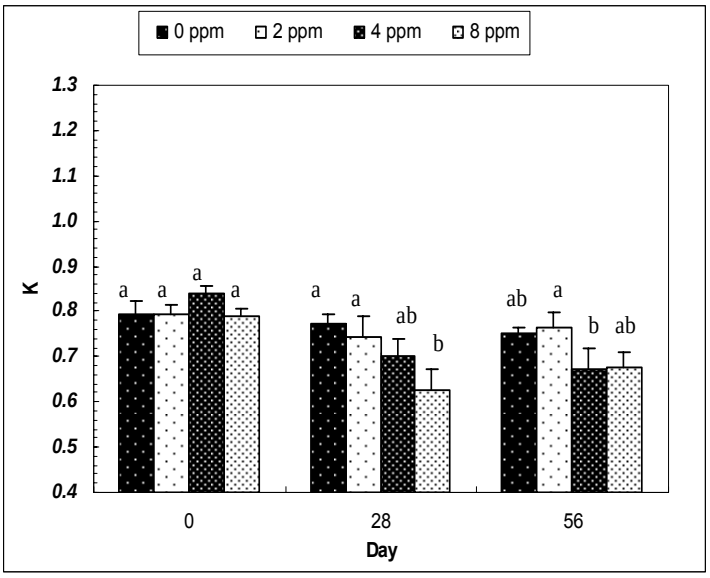
[a] This prepares 1000 g (1 kg) of diet with a calculated crude protein concentration of 32.7 %. Prepare feed in 2-4 kg batches.
[b] USB: USB Corp., Cleveland, OH
[c] Williams and Briggs mineral premix (Purina Mills Test Diets, Richmond, IN).
[d] Vitamin premix supplies per kg of diet the following: vitamin A, 5500 IU; vitamin D3, 1835 IU; vitamin E, 110 IU; vitamin K, 7.3 mg; thiamin, 8.4 mg; riboflavin, 22 mg; pyridoxine, 18.4 mg; pantothenic acid, 58.7 mg; niacin, 36.7 mg; biotin, 2 mg; folic acid, 3.7 mg; vitamin B12, 0.018 mg; choline, 2327 mg; selenium, 0.1 mg.
[e] L-ascorbyl 2-polyphosphate (Stay-C, Hoffman La Roche, Nutley, NJ, USA, 25% ascorbic acid active)(Manning et al 2003)

Figure 1. Antibacterial activity in ug/ml (mean ± SE) in the skin of channel catfish fed 0, 2, 4, or 8 ppm ochratoxin A.



Different letters within a sampling day indicate significant differences between treatment groups (ANOVA, p <0.05). N = 9 fish per treatment group per sampling time.

Figure 2. Condition factor of channel catfish fed 0, 2, 4, or 8 ppm ochratoxin A.



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