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Copper Exposure and Ciliary Function in Gill Tissue of *Mytilus californianus*

John R. Smith*

Mark O. Hatfield Marine Science Center, Marine Science Drive, Newport, OR 97365

Because of global distribution, large size, and sedentary life style, mussels have been used in programs that monitor pollution in various marine environments worldwide (Goldberg et al. 1978). A major category of marine pollutants is metals. Consequently, the bioaccumulation of metals in mussels and other species of bivalves has received attention (Zaroogian et al. 1983; Viarengo et al. 1981; Julshamn et al. 1983). Unfortunately, the biological consequences to bivalves of low-level metal exposure are unclear thus allowing only conjecture about correlations between body burden values and toxicity.

In this study, the biological effects of chronic, sublethal exposure to a metal (copper) on a marine bivalve, Mytilus californianus, were investigated. The biological unit of interest was the function of cilia associated with gill structures. Effects of copper exposure on ciliary activity were assessed using three parameters: 1) The ability of intact animals to remove suspended algae from seawater; 2) cilioregulatory neurotransmitter levels, dopamine (DA) and serotonin (5HT), in the visceral ganglia; and 3) characteristics of putative receptor sites for DA and 5HT in gill tissue.

MATERIALS AND METHODS

Mussels, Mytilus californianus, were collected from the rocky, intertidal shoreline at Yaquina Head, Newport, Oregon, and held in an indoor holding tank supplied with a constant flow of filtered/UV-treated seawater (3 L/minute; 11°C). After a 12-day equilibration period, shell debris was removed, and the mussels were divided into four treatment groups (six animals per group). Each group of animals was placed in a 1,000 ml beaker equipped with an airstone and submerged in a large holding tank receiving 3 L/minute of raw seawater. On the following day, the beakers were removed from the tank, emptied, and raw seawater containing 0, 10.0 ppb, 1.0 ppb, or 0.1 ppb of copper was substituted. The mussels remained in the copper-contaminated seawater for 24 hours during which time the temperature was

*Correspondence and reprint requests: Department of Pharmacology and Toxicology, School of Dentistry, Oregon Health Sciences University, 611 S.W. Campus Drive, Portland, Oregon, 97201

maintained by partial immersion in the larger holding tank. Temperatures through the course of the experiment ranged from 8°C to 12°C with an average of 10.5°. At the end of the 24-hour exposure period, the beakers were emptied, the mussels rinsed with raw seawater, and the beakers submerged again in the main holding tank for 24 hours. This alternating exposure regimen was continued for 62 days.

Animals were fed the unicellular algae Pseudoisochrysis paradoxa approximately twice a week during raw seawater exposure periods. None of the experimental animals died during the course of the experiment.

One day after the last period of raw seawater exposure, the mussels were tested for their ability to clear unicellular algae from seawater. Each animal was placed in 500 ml of seawater containing a known amount of algae (1.52 - 1.91 million cells/ml). At ten-minute intervals, a small aliquant of the seawater (0.5 to 5.0 ml) was sampled and algae concentration determined using a Coulter counter. After the last sample was taken, the animal was returned to the holding tank. The total number of algae cells remaining in the seawater was calculated for each sampling period, and this information was converted to percentage of algae cells remaining. The data were analyzed using student T-tests with an alpha of less than 0.05 as criteria for significance.

On the day following the functional tests, all animals were sacrificed and the two visceral ganglia from each mussel were removed and manually homogenized with glass tissue grinders in 0.5 ml of a 95:5 solution of 0.2 N citric acid-dibasic sodium phosphate and spectrographic-grade methanol containing N-methyldopamine as an internal standard. This solution also served as the mobile phase in later chromatographic procedures. The homogenates were centrifuged for two minutes at 7,000 X g in a cold room (2°C), and the supernatants were transferred to 1-ml syringes and stored at -20°C. Twenty-microliter samples of each ganglionic extract were injected into an Altech high-performance, liquid-chromatography system (Beckman Instrument Co., Redmond, WA) comprised of a 40 X 3.5 mm LiChrosorb C-18 guard column (MC/B Manufacturing Chemists, Inc., Cincinnati, OH) and a 250 X 4.5 mm Ultrasphere C-18 separation column (Beckman Instrument Co., Redmond, WA). The flow rate of the mobile phase was 1 ml/minute, and all separations were done at room temperature (22°C). DA and 5HT levels were determined with an electrochemical detector (Bioanalytical Systems, West Lafayette, IN) by using a glassy-carbon electrode set at a potential of +0.6 V versus the reference electrode. The electronic controller was set at 5 nA/V, the recorder set at 1 V full scale. Identification of DA and 5HT was based upon retention times of standards, and quantification of amines was accomplished by analysis of peak height relative to the internal standard (Saraswat et al. 1981). The student t-test was used for statistical evaluations. An alpha of 0.05 was the minimum criterion for statistical significance.

The gill tissue from each animal (approximately 1 gm, wet weight) was minced and then disrupted in a teflon/glass tissue grinder (20 strokes) containing 10 ml of a chilled buffer solution of 50 mM TRIS, 10 mM ethylenedinitrilotetraacetic acid (EDTA), 0.1 mM phenylmethylsul-

phonyl fluoride, and 6 mM ascorbic acid (pH = 7.4 at 4°C). The homogenate was centrifuged for 15 minutes at 1,000 X g in a cold room (2°C), and the supernatant was filtered through sterile gauze. The filtrate was divided into aliquants and centrifuged for 10 min. at 50,000 X g (5°C). The pellets were rinsed gently with 5 ml of cold assay buffer (50 mM TRIS, 5 mM EDTA, 6 mM ascorbic acid, 10 μM pargyline; pH = 7.4 at 4°C), resuspended in 10 ml of fresh assay buffer, and centrifuged for ten minutes at 50,000 X g (5°C). The pellets were resuspended in chilled assay buffer, pooled, and the protein concentration of the suspension was determined (Lowry et al. 1951). After all tissue suspensions were adjusted to yield 150 ug protein/0.2 ml, they were stored at -25°C. Later, the tissue was thawed and preincubated at room temperature for 20 minutes and placed in an ice bath for two hours. Then 0.2 ml of the tissue suspension was added to 0.4 ml of assay buffer containing 0.2 nM ^{3}H-spiroperidol (New England Nuclear, Boston, MA) and 0, 10^{-10}M, 10^{-9}M, 10^{-8}M, or 10^{-7}M d-butaclamol. Triplicate samples of each concentration of d-butaclamol were incubated for 45 minutes in an ice bath. The assay was terminated by adding 5 ml of chilled assay buffer to each sample and pouring the diluted samples over glass-fiber filters (Whatman GF/B) subjected to 18" Hg vacuum pressure. The filters were washed twice with 5 ml of chilled assay buffer, transferred to counting vials, and allowed to air dry overnight. The next day, 10 ml of Ready Solv-EP scintillation fluor (Beckman Instrument Co., Redmond, WA) was added, and, after the filters became saturated (over six hours), radioactivity was determined at 38 per cent efficiency using an LS8000 liquid scintillation spectrophotometer (Beckman Instrument Co., Redmond, WA).

RESULTS AND DISCUSSION

The cumulative performances of the four treatment groups in the algae clearance test are listed in Table 1.

Table 1. Percentage values (± S.E.M.) of the initial concentration of algae cells still remaining in suspension at various time intervals after addition. Initial concentrations range between 1.5 and 1.9 million cells per ml.

Treatment	n	%Algal Cells Remaining			
		10 min	20 min	30 min	40 min
Control	6	72.8 ± 4.3	49.6 ± 3.6	11.3 ± 3.3	1.2 ± 0.4*
10ppb	6	86.0 ± 4.1 Δ	26.6 ± 3.6 ∇	12.2 ± 1.8	3.7 ± 0.9
1.0ppb	6	79.8 ± 3.6	23.2 ± 2.6 ∇	10.5 ± 0.8	1.9 ± 0.7
0.1ppb	6	86.6 ± 3.6 Δ	15.3 ± 1.3 ∇	8.7 ± 1.1	2.5 ± 0.8

* N = 5
Δ Significantly greater than controls ($P \leq 0.05$)
∇ Significantly less than controls ($P \leq 0.05$)

During the first ten-min. sampling period there was a delay in algae clearance by the treated animals compared with controls. This difference was statistically significant in the groups treated with 10 ppb and 0.1 ppb of copper, but the relationship failed to show a clear dose-dependency. A more dramatic alteration in clearance pattern occurred during the second sampling period: All treatment groups showed significant increases in filtration activity over the control group. This response pattern was clearly dose-dependent, albeit inverse. Subsequent samplings at 30 and 40 minutes showed no differences in algae percentages between treated and control groups suggesting that copper exposure preferentially influences the most rapid phase of algae clearance.

The average concentrations of the ganglionic cilioregulatory transmitters DA and 5HT found in the different treatment groups are listed in Table 2.

Table 2. Concentration of dopamine and serotonin in visceral ganglia of copper-exposed and control mussels after clearance function tests. Values are means ± S.E.M.

Treatment	n	Dopamine*	Serotonin*
Control	6	77 ± 25	303 ± 70
10ppb Cu	5	83 ± 19	281 ± 26
1.0ppb Cu	6	190 ± 34$^{\nabla}$	361 ± 49
0.1ppb Cu	6	220 ± 40$^{\nabla}$	388 ± 57

* pmoles/ganglia pair
$^{\nabla}$ Significantly greater than control

Copper treatment led to a rise in DA content in the visceral ganglia that was statistically significant in the 1.0 ppb and 0.1 ppb treatment groups. As with the clearance data, there was an inverted dose-response relationship, i.e., the magnitude of the effect increased with decreasing levels of copper exposure. A similar pattern was evident in the 5HT responses as well, but no statistical differences could be demonstrated.

Table 3 lists the effects of copper exposure on saturable, high-affinity binding of ^{3}H-spiroperidol in crude membrane preparations of gill tissue from the test animals. No significant differences were evident; however, there was a trend toward a decrease in ^{3}H-spiroperidol binding sites, and this response did display the same inverse dose-response relationship seen previously.

Table 3. Estimates of the maximum number of ^{3}H-spiroperidol sites. Values represent fentamoles (10^{-15} moles) of (3)H-ligand per mg of gill protein.

Treatment	n	(^{3}H)Spiroperidol Bound
Control	5	6.9 ± 1.6
10ppb Cu	5	6.9 ± 2.5
1.0ppb Cu	5	4.4 ± 0.8
0.1ppb Cu	5	3.8 ± 0.5

fmoles/mg protein

This study demonstrates that intermittent exposure of marine mussels to low levels of copper can lead to changes in their functional and neurochemical characteristics.

Little is known about the effects of toxicants upon the ability of bivalve molluscs to filter particulates from their environment. Abel (1976) reported that copper exposure resulted in a dose-dependent decrease in filtration rate in the marine mussel Mytilus edulis. This is in apparent conflict with the results of this study. Abel, however, employed an acute exposure regimen with copper concentrations ranging from 20 to 10,000 times greater than those used here. Possibly the increase in clearance rate seen after chronic, low-level exposure represents an early sign of stress while the clearance deficit described by Abel is indicative of a more advanced stage of stress. If true, this implies that a prolonged exposure to low-level copper would eventually lead to clearance deficits.

An alternate explanation is suggested by the cut-off times of these two studies. The conclusions of the earlier study were based upon filtration rates established over the first ten to 12 minutes of the test. In the present study, a significant delay in clearance in two treatment groups was also evident in the first ten minutes. This agreement, however, turns to conflict in the second ten-minute period when an increase in clearance was seen. Possibly if clearance had been monitored beyond the 12 minutes in the Abel study, increased clearance might also have been seen.

The mechanism by which copper alters filtration in mussels is unknown. The inhibitory effects of high copper concentrations may be related to reduced oxygen consumption. Copper concentrations of 0.25 - 0.39 mg/L can reduce oxygen consumption 12 to 50 per cent in M. edulis (Scott et al. 1972; Dehaye et al. 1975). Since the copper concentrations used in the present study are well below those that alter oxygen consumption, the effects appear to be associated with other actions of copper. Since ciliary activity in Mytilus sps. is influenced by aminergic nerves projecting from the visceral ganglia (Aiello, 1974; Paparo et

al. 1970), the fact that copper exposure leads to an increase in dopamine in these ganglia (Table 2) suggests that the observed functional changes could be a result of neurochemical changes. Dopamine, however, can be both cilio-inhibitory and cilio-excitatory in mussels (Paparo et al. 1970; Malanga et al. 1981) which complicates speculations about causal relationships. Nevertheless, the general observation that chronic, low-level exposure to copper can alter a neurotransmitter system intimately involved in ciliary activity may prove to be an important step toward ultimately understanding the mechanism of toxicity of copper and other metals in bivalve species.

In situations where neurotransmitter levels are changing, it is not uncommon for their specific receptors to undergo density changes (Bylund, 1979). This potential interaction was monitored in copper-treated mussels through radioligand binding studies using the DA-antagonist ^{3}H-spiroperidol, a ligand used routinely to estimate DA-receptor sites (Seeman, 1980). ^{3}H-spiroperidol can bind with high affinity and stereospecificity to dopamine and serotonin binding sites in gill tissue of Mytilus californianus (Smith, 1983). As illustrated in Table 3, there is a definite, dose-dependent trend toward reduced densities of ^{3}H-spiroperidol binding sites in copper-exposed animals. This may be a compensatory reduction in response to elevated DA levels in the gill tissue which may be implied by the elevated ganglionic DA levels.

^{3}H-spiroperidol binding changes may also be due to changes in serotonin binding sites since ^{3}H-spiroperidol can also bind with high affinity to these sites (Seeman, 1980). Although the protocol used makes it impossible to dismiss this alternative, the fact that copper exposure failed to significantly alter serotonin levels in the ganglia suggests that the dopaminergic nervous system may play a more prominent role in the binding changes.

An inverse dose-response relationship was a recurrent theme in each part of this study. Possibly the presence of copper in the water triggered dose-dependent, physiological defense mechanisms. These might include valve closure, increased mucous production, and production of chelating molecules (George et al. 1979; Viarengo et al. 1981; Manley et al. 1979; Slooff et al. 1983). Complete valve closure was never observed in any animal, and excessive frothing in the containment vessels, indicative of increased mucous secretion, was not evident. With regard to the third possibility, Viarengo et al. (1980), showed that Mytilus galloprovincialis can respond to 80 ppb of copper by producing copper-binding proteins. This observation invites the speculation that the enhanced production of these protective proteins at the higher copper concentrations may explain the inverse relationship between copper concentration and biological responses.

A final question is: Are these effects in mussels manifestations of copper toxicity or simply non-detrimental physiological adaptations? Overt toxicity was not apparent; all animals that started the study survived. In addition, all animals responded normally to tactile stimulation, cleared food material from their environment, and set

byssus threads. Abel (1976) suggested, based on correlations between changes in filtration rates and relative toxicities of a series of compounds, that changes in filtration rate represent a valid parameter of toxicity. Thus, the changes in filtration and neurochemistry reported here for low concentrations of copper may represent early indications of toxicity in individual mussels. Efforts are currently underway to clarify this point.

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