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Tadpole Epithelium Test: Potential Use of *Rana catesbeiana* Histopathologic Epithelial Changes to Evaluate Aquatic Pollution

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Aquatic pollutants promote adaptive response in fish, due to epithelial hyperplasia and mucous hypersecretion of gill surface epithelium (Hughes et al., 1976; Nešković et al., 1996; Lichtenfels et al., 1996; Teh et al., 1997). Amphibians are homebodies and live in habitats that may not be shared by fishes, representing a biological indicator for places such as small water bodies that are often favored as breeding sites.

Formaldehyde (HCHO) is an ubiquitous chemical in man's environment. Sources include commercial production, spontaneous formation by incomplete combustion, offgassing from some formaldehyde derived resins and plastics, and metabolic production in all living cells (Swemberg et al., 1983). It is the simplest and most common aldehyde found in the natural environment as an intermediary in the methane cycle with low background concentration (WHO/Environmental Health, 1986). Since its residue fate from several toxic formulations can potentially contaminate the air, soil and water, it is plausible to propose that it could also be defined as a hazardous waste. Owing to its solubility in water, formaldehyde can be absorbed via skin and gill - preferential targets for noxious agent in aquatic organisms - promoting local and systemic effects (WHO/Environmental Health, 1986).

Biological responses to environmental stimuli are determined by alterations of molecular mechanisms that may induce adaptation or conversely, cellular dysfunction (Adams, 1990; Shugart et al., 1992; Teh et al., 1997). Histopathological changes represent an indicator of cumulative action of exogenous stressors upon a specific organism (Chavin, 1973; Stebbins, 1985). In fact, histopathological alterations have been clearly demonstrated in different aquatic organisms exposed to relatively low levels of water pollution, both in field and laboratory studies (Honrubia et al.,1993; Jonnalagadda et al.,1996; Lajtner et al., 1996; Nešković et al., 1996; Lichtenfels et al., 1996; Teh et al., 1997). Therefore, we propose a short-term test to assess the histopathological effects of low concentrations of formaldehyde on gill and skin of the tadpole of *Rana catesbeiana* (bullfrog), under laboratory conditions.

Quantitative studies of skin and gill epithelia, were made after intoxication (24, 48 and 96 hr) with different concentrations of formaldehyde (0, 0.5 and 2.0 ppm). The results indicated that the tadpole epithelium test (TET), as described in this paper, represents a sensitive and inexpensive tool to evaluate short-term histological responses of an aquatic organism to pollution.

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MATERIALS AND METHODS

Tadpoles of the bullfrog *Rana catesbeiana* - stage 26 to 38 according to Gosner (1960) were purchased from a commercial breeder of São Paulo-Brazil. The animals were held under laboratory conditions (23 ± 1°C, 10:14 LD photoperiod), in a community aquarium containing 12 liters of overnight dechlorinated tap water, for one week prior to experimentation. During this adaptive period, the animals were fed with commercial ornamental fish food (Nutrafish of Nutravit - Brazil). Water was changed twice a week.

All the experimental groups were held under the same laboratory conditions (23 ± 1°C, 10:14 LD photoperiod), in 6 L polypropylene plastic aquaria filled with 3 L of overnight dechlorinated tap water. The animals were exposed to different concentration of sublethal doses (0; 0.5 and 2.0 ppm) of formaldehyde (Vetec - Brazil) diluted in the water. The solutions and the aquaria were changed daily - renewal system (Rand and Petrocelli, 1985). No food was given during intoxication. A total of eighty six animals were randomly chosen to form the experimental groups, designed as described in Table 1.

Table 1. Tadpole's intoxication groups for skin and gill analyses.

Groups	Formaldehyde (ppm)	Skin	Gill	
		Intoxication 48 hr (n)	Intoxication 24 hr (n)	Intoxication 96 hr (n)
Control	0	8	12	
I	0.5	9	12	12
II	2.0	9	12	12

After the intoxication period each test animal was immobilized by being transferred to a dish with water and ice for 15 minutes in order to minimize their metabolic functions, then sacrificed. Samples of the dorsal skin and of the hyobranchial skeleton (Duellman and Trueb, 1986) with the gill were collected, placed in prelabeled vials and fixated with buffer solution of formaldehyde (10%) pH 7,0. After 48 hr fixation, tissues were processed for routine paraffin embedding and histological sectioning (5μm thick slides). The paraffin sections were mounted on glass slide and stained with hematoxylin and eosin (HE).

Morphometric evaluations of the skin and of the internal gill epithelium were performed by the point-counting technique (Weibel, 1963). For this purpose, a grid of 100 points was attached to the eyepiece of an optical microscope. At a magnification of 450X, the areal fraction - relative areal proportion of a structure within a reference compartment – of the skin epidermis and of the gill filament epithelium were determined by counting the points falling on epithelium area in 5 randomly chosen non-coincident microscope fields (1,1 mm of length of basement membrane) of each test animal. For this parameter, the areal fraction of the skin epidermis and the gill filament surrounding epithelium (on the median region of the second and third branchial arches), were measured in coded slides.

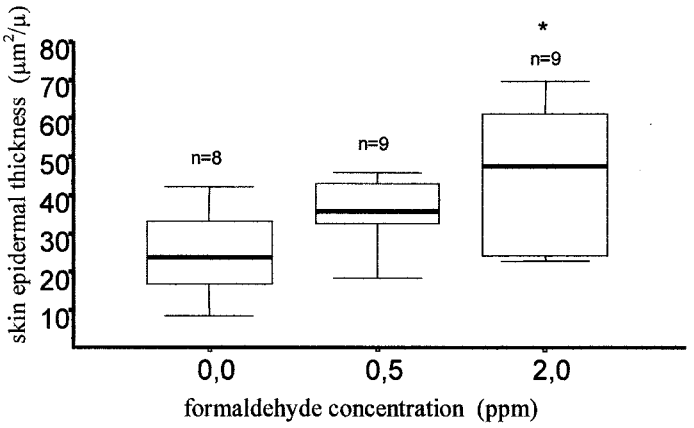


Figure 1. Morphometric evaluation of the skin epidermis areal fraction in control and treated groups. *Statistical data analyses indicate significant difference *(p=0.0238)* between the group treated with 2.0 ppm of formaldehyde in comparison to control group.

Morphometric data were averaged and expressed in terms of means (± SD) to provide a single value for each tadpole. The differences in the mean values among the groups of tadpoles were evaluated by the One Way Analyses of Variance "ANOVA" followed by a post-hoc comparison by Student-Neuman-Keuls for the skin data, while for the gill filament epithelium the Tukey multiple comparison procedure was applied. A p value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

For the skin, the quantitative analyses revealed an increase of the epidermal area proportional to the concentration of the toxicant (Figure 1). Control tadpoles had the *stratum germinativum* with normal morphological structure (Figure 3a). Otherwise, tadpoles exposed to 0.5 ppm showed a tendency for cellular proliferation and those esposed to 2.0 ppm (Figure 3b), showed an epidermal hyperplasia *(p=0.0238)* within the basement membrane and the *stratum corneum*, a common pattern response of epithelia to injury.

The gill epithelium results (Figure 2), showed that the response depends not only on the concentration but also the time of exposure to the toxicant *(p=0.0001)*. Morphological changes were found on the filament surrounding epithelium from treated groups in comparison with control group and among the treated groups. The control tadpoles' filament epithelium had normal morphological structure (Figure 4a). Otherwise, the tadpoles exposed to 0.5 ppm/96 hr (Figure 4b) and 2.0 ppm/24 hr and 96 hr (Figure 4c) of formaldehyde showed a marked hyperplasia of the filament epithelium.

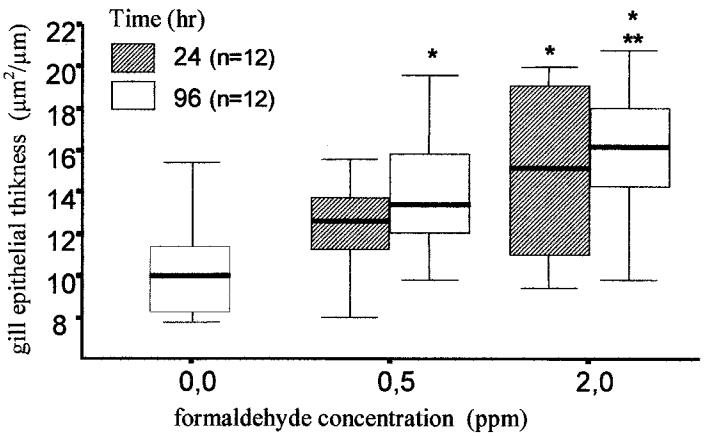


Figure 2. Morphometric evaluation of the gill filament epithelium areal fraction in control and treated groups. *Statistical data analyses indicate significant difference *(p=0.0001)* between treated groups in comparison to control group and among treated groups. ** Significantly different from the group treated with 0,5 ppm (dose)/24 h (time).

Amphibian population throughout the world seems to be declining and some groups are completely disappearing from their native habitats. Though amphibians are in intimate contact with many components of their natural surroundings – water, land and air – it is plausible to propose that their health may be affected by the combined effects of many separate influences on their ecosystem (Blaustein and Wake, 1995). The use of amphibian as a "sentinel" of aquatic pollution has been employed to measure population health and habitat suitability (Larson and Fivizzani, 1994).

The amphibian skin is permeable to water and as such important in the respiration, osmoregulation, and to a limited degree, thermoregulation. (Duellman and Trueb, 1986). The outermost layer of the epidermis, the *stratum corneum*, consists of a single layer of flattened and keratinized cells. Underlying the *stratum corneum* is the *stratum germinativum* of columnar cell separated from the dermis by a basement membrane (Duellman and Trueb, 1986). The gill consistes of four pairs of gill arches, each one with two rows of filaments highly branched, giving rise to a variable numbers of finger-like projections (Burggren and Mwalukoma, 1983), with a distinct overlying epithelium. Considering the high degree of contact with the external milieu of the skin and gills, we reasoned that quantitative pathological evaluation of the degree of epithelial hyperplasia in these locations could be a good indicator of the response to environmental stress. In order to test this hypothesis, the objective of the present study was to evaluate the pattern of skin and gill epithelium short-term response of tadpoles to a soluble toxic agent, under laboratory conditions. For this purpose, we used formaldehyde as the injuring agent and assessed the epithelial response within a short period of time. The basic idea was to develop a rapid and efficient test to assess the toxicity of a water environment.

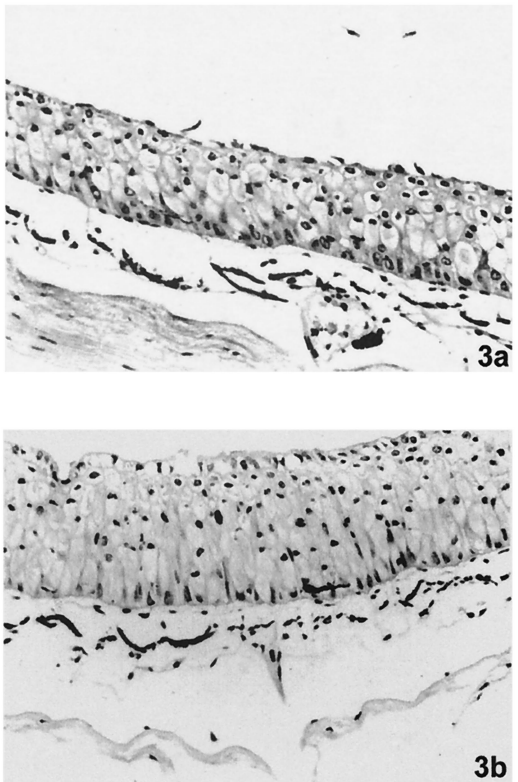
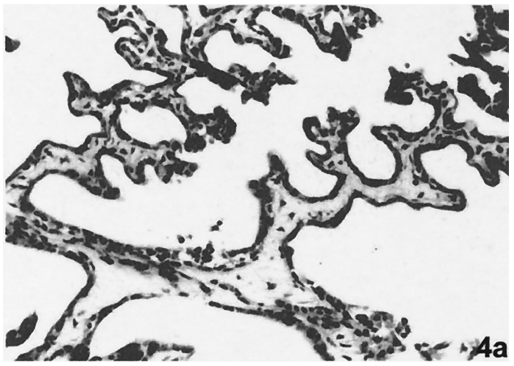
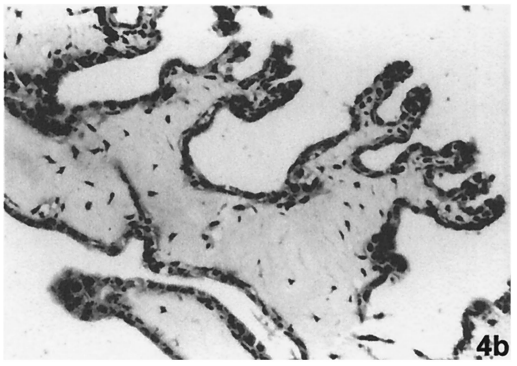


Figure 3. Vertical histological sections of *Rana catesbeiana* tadpole dorsal skin (HE). (a) normal epithelium (200X). (b) Epidermal hyperplasia of a tadpole treated with 2.0 ppm of formaldehyde, 48hr (200X).





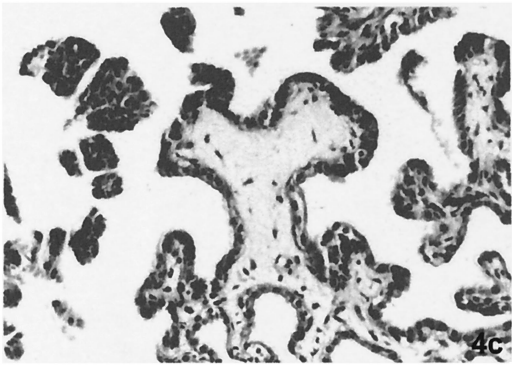


Figure 4. Horizontal histological sections of *Rana catesbeiana* tadpole internal gill (HE). (a) normal filament epithelium (200X). (b) Filament epithelial hyperplasia of a tadpole treated with 0.5 ppm of formaldehyde, 96 hr (200X). (c) Filament epithelial hyperplasia of a tadpole treated with 2.0 ppm of formaldehyde, 96 hr (200X).

In the present study, we found significant morphological changes of skin and gill filamental epithelium, exhibiting a dose-response pattern, detected at low deleterious levels and statistically verified within a short period of time. The histopatological changes of the skin and gill in adaptation to formaldehyde exposure, support the use of the quantification of the hyperplasia of the epidermis and gill surrounding epithelium, as a biomarker of histological response to this chemical stressor. In this scenario, histopathology can be considered a sensitive tool to detect environmental pollution, since it reflects somewhat the time-integrated balance of the relationship between the duration and intensity of the exposure and the adaptive capacity of the tissue under analysis. We are not aware of previous studies focusing the short-term response of tadpole epithelium to environmental contaminants. Based on our results, quantitative histopathological studies are inexpensive since they cost about 1/3 of an enzymatic determination in Brazilian diagnoses laboratories. Furthermore, the TET provides information in a very short time, taking about one to train a person to perform reliable measurements and around 5 minutes to read one slide. Because of its wide distribution and resistance and demanding simple laboratory facilities and easy handling with experimental procedures, the *Rana catesbeiana* tadpole may represent an interesting, achievable and sensitive research model to evaluate aquatic pollution.

Considering our results, we think that TET should be extended to other types of toxic substances and may represent a good alternative when toxicological screening is necessary in the aquatic environment.

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