Cell proliferation and growth-associated protein 43 expression in the olfactory epithelium in *Poecilia reticulata* after copper solution exposure

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This study investigated the regeneration in the olfactory mucosa of the teleostean fish *Poecilia reticulata* when returned to dechlorinated tap water after 4-day exposure to 30 μg/L of Cu^{2+}. The regeneration process in the olfactory tissue was examined in fishes at 0, 3, 6 and 10 days of recovery in well water. Jade B staining permitted to evaluate the rate of the damage which was especially extended to olfactory neurons. Immediately after the end of exposure, a massive mitotic activity in the basal region of the mucosa was detected by immunostaining with PCNA. After 3 days of recovery the nuclei of the newly formed cells had already finished their migration to the upper portion of the epithelium, and cellular division was much less intense. Simultaneously, immunoreactivity for the neural growth-associated phosphoprotein GAP-43 increased respect to control levels, revealing that the new differentiating PCNA-positive elements belonged to immature neurons. After 6 days in well water no mitotic activity was detected, while the GAP-43 labelling appeared particularly concentrated in the apical surface of the olfactory epithelium. After 10 days the aspect of the olfactory epithelium was almost identical to the control. The present results suggest that after 10 days regeneration seems to be complete and integrity of the tissue restored. Furthermore, the epithelium reconstitution does not show apparent divergence from other fishes or mammals.

Key words: PCNA, GAP-43, olfactory receptors; regeneration; copper; fish; *Poecilia reticulata*.

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Paper accepted on March 6, 2006

European Journal of Histochemistry
2006; vol. 50 issue 2 (Apr-Jun):141-146

The fish olfactory system is vulnerable to chemical pollutants since olfactory receptory neurons are directly in contact with the aquatic environment. Many studies have dealt with the toxic effects of Cu^{2+} on fish olfactory system (Moran *et al.,* 1987; Saucier *et al.,* 1991; Julliard *et al.,* 1993; Saucier and Astic, 1995; Julliard *et al.,* 1996; Hansen *et al.,* 1999). In particular, histopathological studies have shown that short-term exposure to low Cu^{2+} levels, just above the no-effect value of 15 μg/L (Birge and Black, 1979), induces degeneration of olfactory neurons, which seems to occur very early in the exposure period (Moran *et al.,* 1992; Julliard *et al.,* 1996; Hansen *et al.,* 1999). It is well known (Thornhill, 1970; Moulton *et al.,* 1970; Takagi, 1971; Graziadei and Metcalf, 1971; Graziadei, 1973; Graziadei and Monti Graziadei, 1979) that the olfactory receptors of adult vertebrates undergo a continuous turnover, the new olfactory neurons originating from stem cells of the olfactory epithelium. These cells divide several times and their progeny differentiate into mature sensory neurons (Calof *et al.,* 1998; Zehntner *et al.,* 1998), and this neurogenic capability is essential for the renewal of mature olfactory neurons that degenerate in response to noxious environmental compounds entering the nasal cavity. While in mammals the regenerative process induced by experimental lesions has been thoroughly studied, in fishes it has been less documented.

The aim of this research is to investigate time course and aspects of the regenerative process in specimens of *Poecilia reticulata* after 4 days exposure to 30 μg/L of Cu^{2+} and, finally, to compare results with those obtained in other fishes and mammals.

Materials and Methods

Animals and tissue preparation

Adult specimens of *Poecilia reticulata* were purchased locally from the *Acquario Fossolo*, Bologna, Italy. All procedures were in conformity with the guidelines of the European Communities Council Directive (86/609/CEE), the current Italian legislation for the care and use of animals and with the guidelines of the U.S. National Institute of Health. The Ethic-Scientific Committee of the University of Bologna also approved this study.

Two experimental groups were considered: 1) an exposed group was placed in 12-l tanks of dechlorinated water in which a calculated quantity of a stock solution of $CuSO_4$ was added every day in order to maintain concentrations of 30 μg Cu^{2+}/l; 2) a control group was left in well water. After 4 days of exposure the first experimental group was returned to well water. On days 0, 3, 6 and 10 of recovery eight specimens for each stage were sacrificed.

Four specimens of the control group were sacrificed on day 10.

Fish were anesthetized with 0.1% 3-aminobenzoic acid ethyl ester (MS-222, Sigma Chemical, St. Louis, MO). They were killed by decapitation and their heads immersed in Bouin solution for 24hrs. They were decalcified in 0.25M EDTA in 0.1M disodium phosphate buffer, pH 7.4 for 1 week. After dehydration in graduate ethanol series, the specimens were embedded in Paraplast plus (Sherwood Medical, St. Louis, MO; melting point 55-57 °C), frontally sectioned (6 μm) with Leitz 1516 and mounted on poly-L-lysine-coated slides. The sections were subjected to cresyl violet staining, Fluoro Jade B staining and GAP-43 and PCNA immunodetection.

Fluoro Jade-B staining

Tissue sections were rehydrated in ethanol series. After 15 min in $KMnO_4$ 0.06%, to minimize background, and subsequent rinsing in distilled water, sections were immersed in 0.001% Fluoro Jade staining solution (Chemicon International, Temecula, Ca) (Schmued *et al.,* 1997) in the dark for 30 min. After removing the excess of fluorochrome by rinsing in distilled water, slides were dried at room temperature and then mounted in DPX (Fluka BioChemika, Bucks, Switzerland). The tissue was then examined using a confocal scanner (Leica TCS SP2) with blue (450-490 nm) or blue-violet (420-490 nm) excitation light.

Immunohistochemistry

Mouse monoclonal antibodies against PCNA (1:500; P8825, Clone PC10, Sigma), specific for nuclear proliferation, and mouse monoclonal antibodies against GAP-43 (1:1000; G9264, Clone GAP-7B10, Sigma), a phosphoprotein involved in neuronal differentiation, were used. Sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked with 1% H_2O_2 in 10mM phosphate saline buffer (PBS) pH 7.4. After rinsing in the same buffer, specimens were immersed in antigen unmasking solution (Vector Laboratories, Burlingame, CA) and treated with microwaves for 5 min at 750 Watts. After rinsing, the nonspecific background staining was reduced with preincubation in PBS containing 10% normal goat serum (NGS; Vector), 1% bovine serum albumine (BSA; Sigma) and 0.1% Tween 20 (Merck, Darmstadt, D) for 30 min. The antibodies were diluted in PBS containing 3% NGS, 1% BSA and 0.1% Tween 20. After rinsing in PBS with 0.1% Tween 20, the sections were incubated for 1 hr and 30 min in the secondary antibody: HRP-conjugated goat anti-rabbit IgG (1:100; Vector Laboratories) in PBS containing 1% BSA and 0.1% Tween 20. After rinsing in 0.1 M phosphate buffer, pH 7.4, the sections were treated with diaminobenzidine and counterstained with hematoxylin (Vector Laboratories). The sections were then dehydrated in ethanol, cleared in xylene and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Negative controls were obtained by omission of the primary antibody, replaced by 3% NGS.

The number of PCNA labeling cells was estimated in all the specimens after 0, 3, 6 and 10 days of recovery in well water and compared to the data obtained from the controls. Values were reported as mean and deviation standard (Table 1).

Results

In *Poecilia reticulata*, a paired olfactory organ was located in the dorsal region of the snout. Each olfactory organ consisted of an olfactory chamber in which a series of lamellae forming the olfactory rosette raised from the floor. The lamellae consisted of sensory and non-sensory epithelia. The olfactory receptor neurons, intermingled with supporting

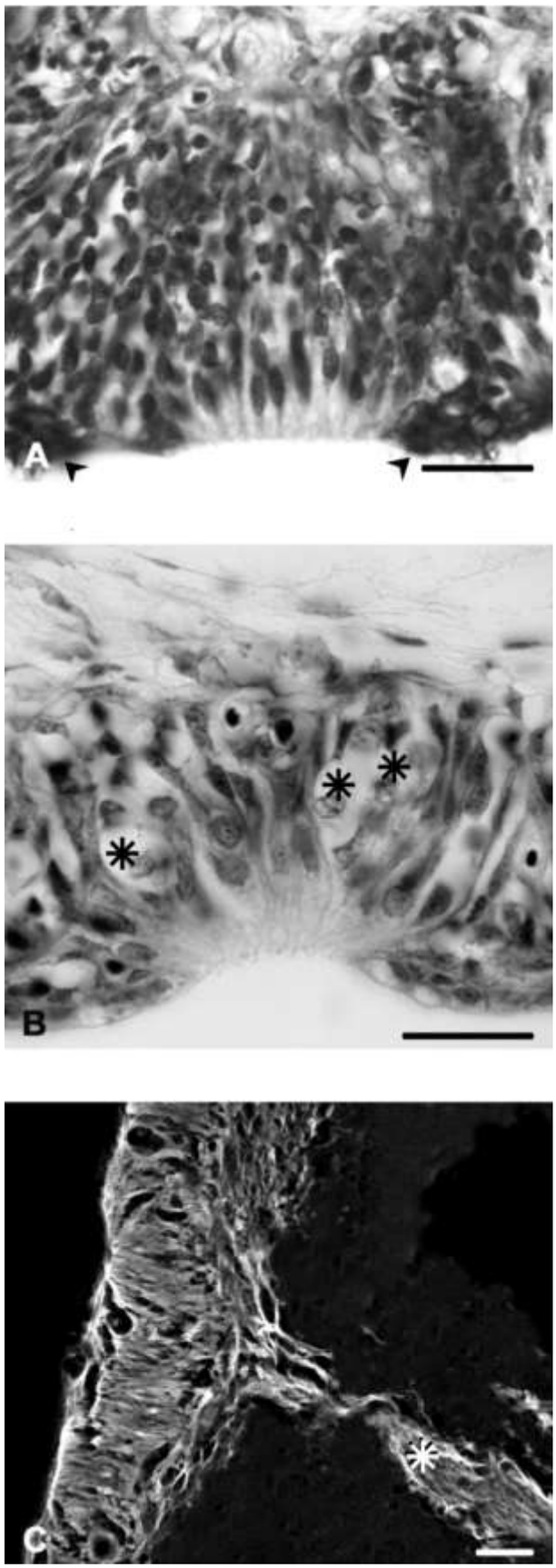

Table 1. Number of PCNA labeling cells in the olfactory epithelium of *Poecilia reticulata*.

Time of recovering in well water	*Mean of the number of PCNA labeling cells*
Time 0	143.2 ± 10.9
3 days	58.8 ± 8.9
6 days	15.8 ± 3.6
10 days	13.5 ± 2.3
Controls	10.4 ± 1.9

cells, were confined to goblet grooves between ridges of non-sensory epithelium (Figure 1A).

After 4 days of exposure to 30 μg/L of Cu^{2+}, the olfactory receptor neurons showed signs of degeneration with the loss of intercellular connections and degeneration of many cells, whereas the supporting cells were preserved (Figure 1B). Fluoro Jade B, which selectively stains degenerating neurons, confirmed that the degeneration pattern was extended to the entire epithelium (Figure 1C). In particular, the epithelial apical region and some axonic fibres in the olfactory nerve were intensely labeled.

PCNA-immunocytochemical detection

After 4 days of exposure to 30 μg/L of copper, at time 0, a lot of PCNA-positive nuclei were present in the olfactory epithelium. They were mainly located in the basal region of the mucosa (Figure 2A), even if some labeled nuclei were localized in the middle layer of the epithelium. The number of the PCNA stained nuclei found at this stage reached the highest value when compared with the other recovering periods (Table 1).

After 3 days of recovery in well water the number of PCNA-positive cells decreased (Table 1) especially in the basal layer of the epithelium (Figure 2B). An increased staining was visible in the immediate upper layer, with a number of positive nuclei comparable to the number present in the lower region, even if the signal appeared less evident.

A further decrease of the anti-PCNA labeled

Figure 1. Olfactory epithelium of control *Poecilia* (A) and of fish exposed to copper (B-C). A: Cresyl violet staining of a goblet olfactory groves of a control specimen. Arrowheads: ridges of non-sensory epithelium. B: Olfactory epithelium of fish exposed to 30μg/L for 4 days. Some degenerating cells (asterisks) are evident. C: Fluore Jade B staining of degenerating receptor cells in the olfactory mucosa and axons in the olfactory nerve (asterisk) in fish exposed to 30μg/L. Scale bars = 20 μm (A, B, C).

nuclei was detected after 6 days in well water, and confirmed after 10 days (Table 1 and Figure 2C), a situation similar to what was observed in control sections (Figure 2D).

GAP-43-immunocytochemical detection

This phosphoprotein, during development, is normally present in all cytoplasmic districts.

After 3 days of recovery in well water, the epithelium appeared intensely labeled by anti-GAP-43 (Figure 2E). At this stage the immunostaining appeared mainly restricted to the perinuclear cytoplasm of immature neurons and, in particular, in the proximal tract of the dendritic extention.

After 6 days of recovery the GAP-43 labeling shifted towards the apical pole of the epithelium which was intensely marked (Figure 2F).

On day 10 of immersion in well water the immunocytochemical reaction still gave a positive even if less intense signal, especially at the free surface of the mucosa (Figure 2G). The labeling was similar to control epithelium whereas the reaction was mostly localized in the apical olfactory regions (Figure 2H).

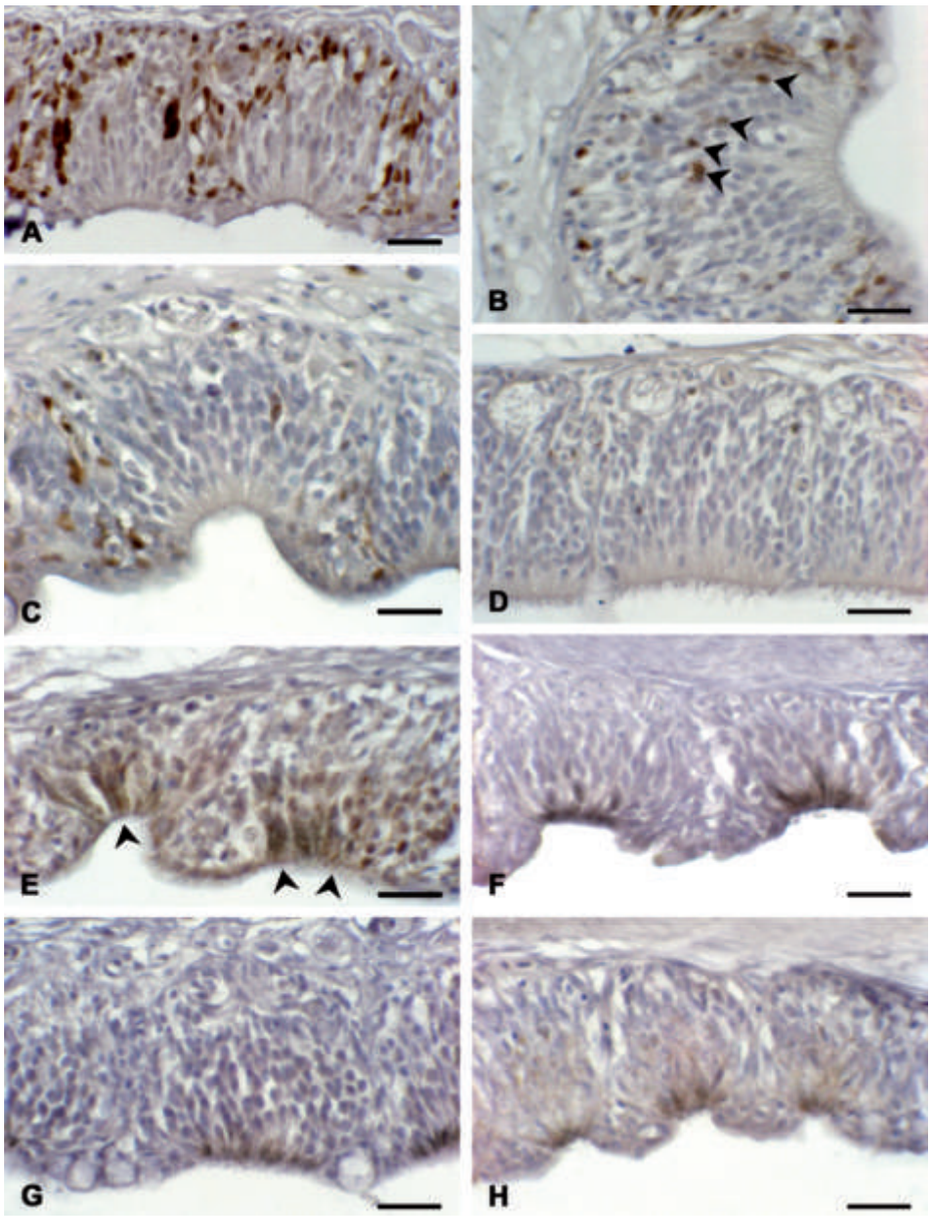

Figure 2. Immunohistochemical staining for PCNA (A, B, C, D) and for GAP-43 (E, F, G, H) in the olfactory epithelium during recovering after 30µg/l copper exposure. A: PCNA-positive nuclei in the basal region of the olfactory mucosa at time 0. B: After 3 days of recovery, PCNA-positive cells decreased in the basal layer of the epithelium and some labeled cells are visible in the immediate upper layer (arrowheads). C: After 6 days of recovery, scarce PCNA-positive cells are detectable in the olfactory epithelium. D: anti-PCNA control section. E: GAP-43 labeling after 3 days of recovery. Many receptors are intensely stained (arrowheads). F: After 6 days of recovery, the GAP-43 labeling is mainly located in the apical region of the epithelium. G: After 10 days of recovery, the GAP-43 labeling is located on the free surface of the epithelium. H: GAP-43 labeling of control fish. Scale bars = 20 µm (A, B, C, D, E, F, G, H).

Discussion

The results of this study provide further evidence of the toxic effects of Cu^{2+} low-levels on fish peripheral olfactory organ. The olfactory epithelium of *Poecilia reticulata* appeared seriously injured by 4 days exposure to 30 μg/L of Cu^{2+}, as shown by Fluoro Jade B staining.

In the olfactory epithelium the mature neurons down-regulate the proliferation of new receptors by neuronal progenitors in order to maintain the size of their population at a defined level (Wu *et al.,* 2003). The experimental lesion removes the inhibition and activates the regenerative response of the germinative layer of the epithelium whose first step is an increased basal cell proliferation in agreement with what was reported by Ducray *et al.* (2002) and Min *et al.* (2003) in mice. The present study showed that at the end of exposure the mitotic activity in the basal layer was higher than the normal turnover followed by a progressive dropping of the PCNA labeling to control levels. Immuno-stained nuclei in the upper layer after 3 days of recovery demonstrate that, after mitosis, these new elements migrate away from the basal compartment while they are differentiating. These findings are in agreement with those obtained by BrdU-labeling in lacustrine sockeye salmon (Yanagi *et al.,* 2004) and ^{3}H-thymidine injected mice (Mackay-Sim and Kittel, 1991). The weaker intensity of the labeling showed at this stage was probably due to a reduced expression of the antigen in the days after mitosis.

Moreover, we observed that control fish showed a weak GAP-43 immunoreactivity in the apical surface of the olfactory epithelium, probably due to some immature elements that completed the maturation to counterbalance mature receptor cell death in normal turnover. Yet, after 3 days of recovery the reactivity was much more evident and not only diffused in the apical region of the epithelium. The high number of differentiating cells belonging to immature neuron population suggests that low doses of Cu^{2+} specifically induce degeneration of olfactory primary neurons. On the contrary, the other cellular elements of the epithelium are less involved as already reported in *Salmo trutta* (Moran *et al.,* 1992) and in *Oncorhynchus mykiss* (Julliard *et al.,* 1993, Julliard *et al.,* 1996). On the 6th day of recovery in well water, terminal knobs differentiated. Knobs differentiation is the final step of maturation of the dendritic branch.

At this stage the cells originated by induced proliferative activity seem to have completed their development. At the same time, mitotic activity in the basal layer decrease reaching the value of controls. Hence, the regeneration can be considered complete. Therefore, the new olfactory cells need about 6 days to develop while they require about 10 days to reach a complete maturation as showed by a control-like labeling of the differentiating factor GAP-43. These data agree with the observation of Julliard *et al.* (1996) during continuous copper exposure. The authors observed two peaks of cell death, the first after 5 days of exposure and the second after 15 days, suggesting that a 10 day period is needed for the receptors to mature.

It is known that the extent of the induced lesion can differ among species, even when subjected to the same conditions of treatment (Hansen et al, 1999; Ducray *et al.,* 2002), and, as a consequence, the time for olfactory epithelium reconstitution can show a species-specific delay. However, there is no apparent divergence in the time of differentiation for basal cells to mature olfactory receptor neurons both in *Poecilia reticulata* and in other fishes as well as in mammals (Graziadei and Monti-Graziadei, 1979; Michel *et al.,* 1999). Moreover, the receptor differentiation period of about 1 week is observed both in olfactory organs subjected to axotomy (Moran *et al.,* 1992) and in the organs physiological turnover (Yanagi *et al.,* 2004). Therefore, present observations underline that the type of experimental injury does not seem to influence the time-course of development of new receptor cells.

Acknowledgments

This study was supported by grants from the University of Bologna, (Molecular signal and mechanisms in cellular survival).

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