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Original Article

Cadmium induced histopathology in the olfactory epithelium of a snakehead fish, *Channa punctatus* (Bloch)

Debraj Roy, Debasree Ghosh, Dipak Kumar Mandal*

Department of Zoology, Visva-Bharati University, Santiniketan-731 235, West Bengal, India.

Abstract: Histopathology on the olfactory organ of a snakehead fish, *Channa punctatus* (Bloch, 1793) were assessed after exposing the fish to 2.5 mg/L and 5mg/L of $CdCl_2$ for 15 days, 30 days and 45 days. Cellular organization of the epithelium was affected severely with degeneration of sensory and supporting cells and hyperplasia of basal cells and mucous cells. Mucous cell proliferation indicates the upregulation of mucous secretion to protect the epithelium from toxic effect of cadmium. The olfactory epithelium was endowed with the multipotent basal cells which differentiate into sensory cells, supporting cells and other cell types of the epithelium during normal cells turn over and in the event of cell death. However, due to cadmium exposure proliferating basal cells failed to differentiate into normal cells and the undifferentiated proliferated cell formed lump and intraepithelial lesion altering the composition of the entire epithelium. Present study indicates that in prolonged exposure to cadmium chloride olfactory functions of the fish might be impaired due to loss of all sensory cells.

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Introduction

Fish are dependent on the olfactory sense for discriminating a wide array of odorous molecules that are involved in survival-linked behaviors such as food searching, predator avoidance, migration, reproduction and parental care (Hara, 1992). Structural organization of the olfactory organ has been described in *Channa punctatus* (Mandal et al., 2005) and in other fishes (Chakrabarti and Ghosh, 2010). Olfactory organs of fish are particularly vulnerable to aquatic toxicants since their receptor cells are directly exposed to the environment and any damage in the olfactory system may adversely affect to the survival of fish species. In an aquatic environment, chemical wastes including heavy metals are common pollutants. Mercury causes severe histological damage in the kidney (Ghosh and Mandal, 2012) and in the olfactory system of fish (Ghosh and Mandal, 2013). Contaminants altered normal olfactory mediated behaviors by modifying odorant perception (Tierney et al., 2010). Copper exposure causes ciliary loss and cell death in the olfactory epithelium of fish (Moran et al., 1992; Julliard et al., 1996). Aluminum also causes damage to the olfactory epithelium of fish (Klaprat et al., 1998). Cadmium is known toxic heavy metal and common contaminants in aquatic system. Cadmium and other divalent metal ions were taken up by olfactory sensory neurons of both rodent and fish and then transported through axon towards the olfactory bulb (Tjälve and Henrickson, 1999). Cadmium inhibited Ca^{++} influx by interfering Ca-ATPase activity (Verbost et al., 1989). Cadmium interferes with cellular signaling or suppresses the apoptotic response, which doubtlessly explains the widely recognized carcinogenic properties of this heavy

* Corresponding author: Dipak Kumar Mandal
E-mail address: dkmandal.vb@gmail.com
Tel: +919474166574

metal (Shimada et al., 1998). Cadmium causes histopathology in the liver, kidney and other organ system of fish. The present work has been designed to study the effect of cadmium on the histopathology of the olfactory epithelium of *Channa punctatus*.

Material and methods

Maintenance of test animal: Freshwater snakehead fish, *Channa punctatus* (Bloch, 1793) (22 to 27 cm in length and 100 to120g in weight) were procured from the freshwater ponds at Santiniketan (23.68^{o} N 87.68^{o} E), West Bengal, India and 120 adult, healthy fish were acclimatized under laboratory conditions for 15 days. Fish were fed *ad libitum* with commercially available feed pellet and water was changed daily. Physico-chemical parameters of water was analysed following the methods of APHA (2010). The water parameters in the experimental aquarium were temperature 20-22 ^{o}C, pH 7.2; dissolved oxygen 4 to 4.2 mg/L; total alkalinity 160 mg/L and total hardness 166 mg/L. No mortality was observed during this period. Fish were maintained and experiments were conducted following the guideline of animal ethics committee, Department of Zoology, Visva-Bharati University, India.

Experimental design: After acclimatization fish were selected randomly and transferred to experimental aquarium. Total nine aquarium with 10 fish and 30 liters of water in each were taken for experiments. Experimental aquarium were grouped into 3 sets containing 3 aquarium in each set. In each set of 3 aquarium, one aquarium was kept as control and other two were contaminated with 2.5 mg/L and 5 mg/L of $CdCl_2$, respectively. In the first set, fish were exposed for 15 days and in the second and third sets fish were exposed for 30 days and 45 days respectively. The two sublethal dose of $CdCl_2$ were selected for the experiments as 5% and 10% of the 72 hour LC_{50} value of $CdCl_2$ for the fish (72h LC_{50} value 50.05 mg/L) which was determined following probit analysis method (Finney, 1977). Fish were kept under continuous aeration. Water was changed and doses were renewed at 2 days interval. After completion of experiment fish were anaesthetized with 100 mg/L MS 222 and sacrificed. Intact olfactory tissues were collected from both control and treated fish and processed for histology.

Tissue preparation: Olfactory tissues were fixed in aqueous Bouin's fluid for 20 hours at room temperature. Fixed tissues were washed in 70% ethanol repeatedly and dehydrated through graded series of ethanol and cleared in xylene. Tissues were then infiltrated in paraffin wax (56-58 ^{o}C, Merck, Germany) for 45 minutes under a thermostat vacuum paraffin embedding bath for 1 hour. Serial thin (4 μm) sections of the paraffin block of the tissue were obtained using a rotary microtome. Six sections in two rows were taken in albumenized glass slide and stretched using a thermostat hot plate. The sections were deparaffinized with xylene, hydrated through downgraded ethanol to distilled water. The tissue were stained with Delafield haematoxylin and counterstained with eosin (1%). Stained slide dehydrated with graded ethanol, cleared in xylene and mounted with DPX.

Histological study: Stained slides were examined, compared to control group and photographs were taken under BX51 Olympus Research Microscope. For each treatment group 10 slides were prepared from the tissue of five fish. Measurement of cell diameter and thickness of epithelium were taken by ocular micrometer. Histopathological alterations in the olfactory epithelium were assessed semi-quantitatively by using score ranging from – to +++ depending on the degree of alteration: (-) no alteration (+) mild alteration, (++) moderate alteration and (+++) severe alteration.

Results

Olfactory epithelium of control fish: Olfactory epithelium (OE) of *C. punctatus* was a thick (30-40 μm) sheet of pseudostratified epithelium consisting of columnar sensory and non-sensory cells, round basal cells and mucous cells. Olfactory epithelium infolded into 18-20 lamellae and formed olfactory rosette. In lamellae, the epithelium enclosed a stromal sheet containing blood vessels, connective tissues and nerve fibers. Basal lamina (BL) separated

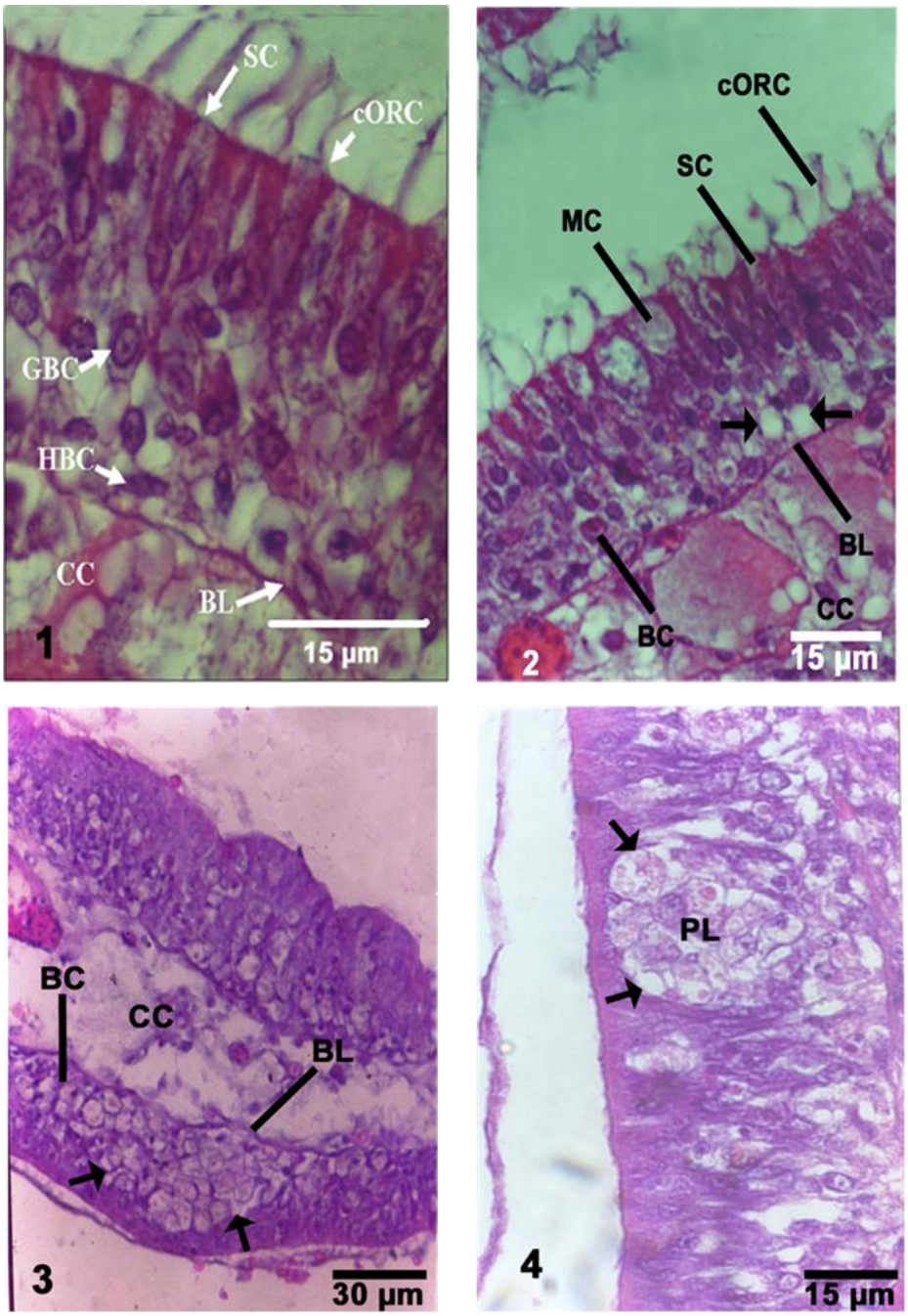

Figure 1. Sensory olfactory epithelium (OE) of control fish showing the normal arrangement of olfactory receptor cells (ORC), supporting cells (SC), globular basal cells (GBC) and horizontal basal cells (HBC). Basal lamina (BL) in between the epithelium and central core (CC) (*Channa punctatus*, H&E).

Figure 2. OE of the fish exposed to 2.5 mg/L $CdCl_2$ for 15 days, showing the proliferation of mucous cells (MCs), normal arrangement of ciliated ORC (cORC) and SCs and a few vacuoles (arrow). BL remained distinct and intact (*C. punctatus*, H&E).

Figure 3. OE of the fish exposed to 5 mg/L $CdCl_2$ for 15 days showing hyperplasia of BCs (arrows). BL distinct and intact (*C. punctatus*, H&E).

Figure 4. OE of the fish exposed to 5 mg/L $CdCl_2$ for 30 days showing altered cellular organization and proliferative lesion (PL). Proliferating cells with large nucleus (arrows). Vacuoles indicated the degeneration of cells (*C. punctatus*, H&E).

stromal sheet from the epithelial cells. Sensory cells were found in the mid lateral side of the lamellae whereas non-sensory epithelium occupied the tip and basal part of the lamellae. Sensory epithelium was composed of ciliated (cORC) and microvillous (mORC) olfactory receptor cells, supporting cells (SC) and basal cells (BCs). ORCs and SCs were arranged in alternate rows. Basal cells were of two

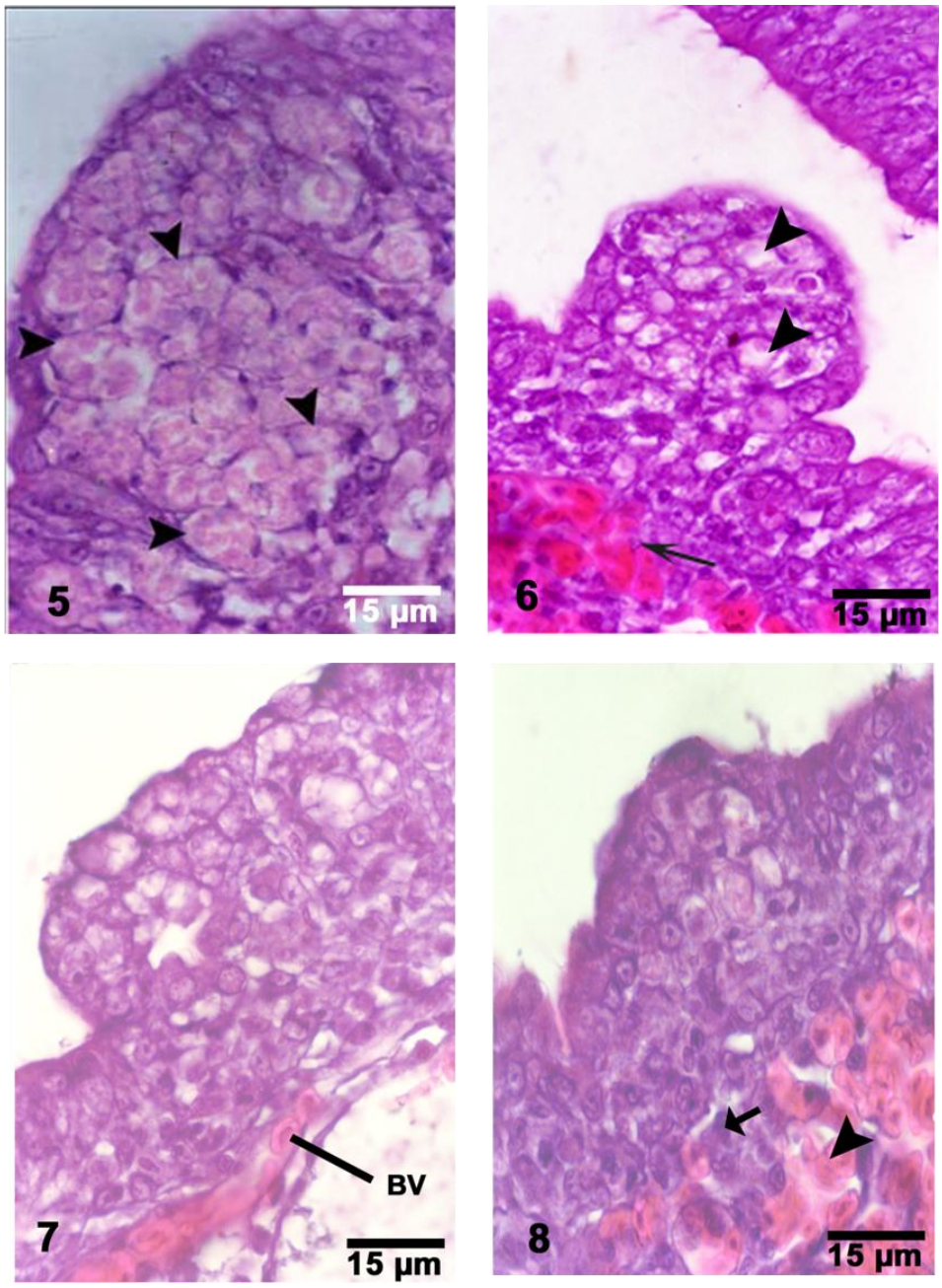

Figure 5. Olfactory epithelium (OE) of the fish exposed to 5 mg/L $CdCl_2$ for 30 days showing the proliferative lesion with large, round undifferentiated cells (arrow heads) and small cells with pyknotic nucleus around the lesion (*C. punctatus*, H&E).

Figure 6. OE of the fish exposed to 5 mg/L $CdCl_2$ for 45 days showing thickening and outgrowth of epithelium, vacuoles (arrowheads), complete degeneration of columnar sensory and non-sensory cells and blood vessels (BV) formation within epithelial cells (*C. punctatus*, H&E).

Figure 7. OE of the fish exposed to 5 mg/L $CdCl_2$ for 45 days showing altered cellular organization. BV within epithelium and above BL (*C. punctatus*, H&E).

Figure 8. OE of the fish exposed to 5 mg/L $CdCl_2$ for 45 days showing extensive ramification of BV (arrowheads) and invasion of BV inside the lesion, disruption of BL, mixing of proliferating cells with the blood cells (arrow) (*C. punctatus*, H&E).

morphological types including globular basal cells (GBC) and horizontal basal cells (HBC) (Fig. 1). Non-sensory epithelium possessed ciliated columnar cells (cNSC), mucous cells (MC) and BC.

Olfactory epithelium after 15 days exposure to $CdCl_2$: Mucous cells hyperplasia was found all over the epithelium. In 2.5 mg/L $CdCl_2$ exposed fish, ORCs, SCs, BCs and BL were as normal as control

Table 1. Semi quantitative scoring of the histopathology in the olfactory epithelium of $CdCl_2$ exposed *Channa punctatus*.

Histopathology	$CdCl_2$ exposure					
	15 days		30 days		45 days	
	2.5 mg/L	5.0 mg/L	2.5 mg/L	5.0 mg/L	2.5 mg/L	5.0 mg/L
Hyperplasia of mucous cells	++	+++	+++	+++	+++	++
Degeneration of sensory cells and supporting cells	-	++	++	++	+++	+++
Hyperplasia of basal cells	-	++	++	+++	+++	+++
Proliferative lesion	-	+	+	++	+++	+++
Alteration in the cellular organization of epithelium	-	+	+	++	+++	+++
Lump formation	-	-	-	+	+	++
Angiogenesis	-	-	-	-	-	++

Score: (-) No alteration, (+) Mild alteration, (++) moderate alteration, (+++) severe alteration.

except a few vacuoles in the basal cell region (Fig. 2). Cell degeneration in sensory and non-sensory epithelium as well as basal cells hyperplasia were found when the fish exposed to 5 mg/L $CdCl_2$. Proliferating basal cells formed cluster along the basal lamina. These cells were oval and larger than the normal basal cells. Cell degeneration were evident with the vacuoles formation Abnormal proliferation of basal cells altered the organization of the epithelium (Fig. 3). Basal lamina and stromal sheet remained unchanged.

Olfactory epithelium after 30 days exposure to $CdCl_2$: Degeneration of sensory and non-sensory cells and hyperplasia of basal cells and mucous cells were observed when fish exposed for 30 days even at low concentration (2.5 mg/L) of $CdCl_2$. Neoplasia of basal cells formed intraepithelial lesions. The proliferative cells were larger (10±1.24 µm) than the normal basal cells (6.66±0.66 µm). Nucleus to cytoplasmic ratio of these cells was higher than the normal basal cells. Columnar sensory and non-sensory cells were completely degenerated and the epithelium was occupied by the proliferating undifferentiated cells. Intraepithelial lesions grew further and formed lumps at an exposure to 5 mg/L of $CdCl_2$ (Fig. 4). A few of these lumps were protruded out of the epithelial surface. Intercellular vacuoles within the lesion indicated the degeneration of proliferating cells. However, no blood vessel formation within the epithelium was found. Basal lamina and stromal sheet were remained unaltered (Fig. 5).

Olfactory epithelium after 45 days exposure to $CdCl_2$: Olfactory epithelium encounter with severe histopathology. Even at 2.5 mg/L of $CdCl_2$ exposure, proliferating cell mass increased the thickness of the epithelium and protruded out of the surface (Fig. 6). Sensory cells and columnar non-sensory cells were completely degenerated. Enlarged intraepithelial lesions were occupied the epithelium at 5.0 mg/L $CdCl_2$ exposure. Angiogenesis within the epithelium and lesion and basal lamina disruption occurred (Figs. 7, 8).

Discussion

This study provided a clear evidence of the toxic effects of $CdCl_2$ on the olfactory epithelium of *C. punctatus*. The structural organization of the epithelium altered due to degeneration of sensory and non-sensory cells and proliferation of basal cells. The change or damage to the olfactory system might strongly influence its functions as well as the fish behavior (Hernadi, 1993; Bettini et al., 2006).

The immediate response of olfactory epithelium to cadmium toxicity was the hyperplasia of mucous cells. Julliard et al. (1993) found that goblet cells increased in number following metal treatment. Increase of mucous cell population was also reported by Dang et al. (1999) in the gill epithelium following exposure to copper. Immunohistochemical study revealed that mucous bound with metallothionein cadmium conjugate took an active role in eliminating the metal from the system (Roy et al., 2012). Therefore, hyperplasia of mucous cells and its enhanced secretion as observed in the present study was a protective mechanism. However, mucous secretion did not succeed to protect the olfactory

epithelium when concentration of the metal and duration of exposure were enhanced.

Cadmium chloride exposure for a short period caused less histological damage to the olfactory epithelium due to its excellent regenerating power. Basal cells differentiate into sensory and non-sensory cells of the epithelium during normal cell turn over and in the event of cell death. However $CdCl_2$ exposure for a long period caused severe histopathology in the olfactory epithelium of the fish including degeneration of all sensory cells. Julliard et al. (1993) observed that sublethal exposure to copper caused specific degeneration of all mature receptor cells as well as numerous immature neurons in the olfactory epithelium of *Oncorhynchus mykiss*. Hansen et al. (1999) showed that the number of receptor cells in the olfactory epithelium significantly reduced following exposure with 15 µg Cu/L. Cell death stimulate the proliferation of basal cells to repair the damage. Basal cell hyperplasia and numerous clusters of undifferentiated basal cells were found in the olfactory epithelium of cadmium exposed fish groups. Similar result was found by Julliard et al. (1993) who observed numerous clusters of cells in the basal region following the exposure to copper. Bettini et al. (2006) proved that low level of Cu^{2+} was responsible for the death of fish olfactory receptor neurons. Cu^{2+} increased the mitotic activity of the basal region of the epithelium. This study revealed that at prolonged exposure to $CdCl_2$, uncontrolled proliferating basal cells and their failure to differentiate into normal sensory and non-sensory cells caused alteration in the normal composition of the olfactory epithelium. Columnar sensory and non-sensory cells were replaced by stratified non-sensory cells. These results indicated that cadmium disrupted the normal cell cycle regulation.

In this work, hyperplasia of basal cells, inflammation, proliferative lesion formation and angiogenesis in the olfactory epithelium of cadmium exposed fish indicated that cadmium chloride disrupted normal cell cycle regulation and carcinogenic at prolonged exposure. Several reports supported that cadmium induced cell transformation. Cadmium induced tumour in pituitary, testes and lungs (Waalkes et al., 1999) and induced malignant transformation of human prostate epithelial cells (Achanzar et al., 2001). Dally and Hartwig (1997) observed that cadmium inhibited the DNA damage repairing. Méplan et al. (1999) showed that cadmium suppressed cell cycle regulatory protein, p53 response to DNA damage repairing. This inhibition of p53 function could play a role in cadmium induced cell transformation.

This study revealed that cadmium chloride was toxic to the olfactory epithelium of *Channa punctatus* and at prolonged exposure it caused irreversible damage to the olfactory sensory epithelium that might impair the olfactory function of the fish.

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