Cellular responses in the skin of carp (*Cyprinus carpio*) exposed to copper

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Abstract

The skin of carp, *Cyprinus carpio*, was examined at the ultrastructural level after exposure to 1.6 $\mu$M (100 $\mu$g l$^{-1}$) of copper for different periods, up to 43 days. During the first 7 days, the skin surface became highly undulating and covered by a web of glycocalyx and amorphous mucus. At this period, degenerative pavement cells (both apoptotic and necrotic) were common. During the whole period filament cells from the outer epidermal layers were active in synthesizing secretory vesicles of high electron density. Mitotic activity was enhanced, and was located mainly in the vicinity of club cells. Mucous cells differentiated close to the skin surface, became elongated and synthesized highly electron-dense, probably serous, mucosomes. The latter showed peroxidase activity. Many leucocytes invaded the skin, and chloride cells appeared close to the epidermal surface. Merkel cells were depleted of their secretory vesicles during the first week of copper exposure, but recovered afterwards. In the dermis, fibroblasts became more active in the synthesis and secretion of collagen. The pigment in the melanocytes was dispersed in the first weeks, but this reversed towards the end of the experiment. These changes reflect the sensitivity of the skin of fish to waterborne copper. They are comparable to the effects of stressors such as cadmium, lead, or acid water.

Key words: Epidermis; Dermis; Ultrastructure; Copper pollution; Stress; *Cyprinus carpio*

1. Introduction

Pollution of aquatic systems with heavy metals, including copper, affects fish populations by reducing growth and reproduction, and may eventually impair their surviv-

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Copper exerts a wide range of physiological effects on fish. Exposure to copper stimulates the synthesis of metallothioneins in hepatocytes (Dixon and Sprague, 1981). It causes changes in several blood parameters: elevation of plasma cortisol levels (Schreck and Lorz, 1978) and of plasma glucose and ammonia levels (Laurén and McDonald, 1985) have been reported, as well as a decrease in red and white blood cell counts (Khangarot and Tripathi, 1991). Exposure to copper affects plasma ion levels. In particular a reduction of plasma Na⁺ concentrations has been reported (Reid and McDonald, 1988).

The gills are the most important organs for ion regulation, and the Na⁺ imbalance indicates that they are among the first organs affected by copper (Laurén and McDonald, 1985). Not surprisingly, also morphological changes have been reported for the branchial epithelium after exposure to copper. These changes were mostly of a degenerative nature (e.g. necrosis of epithelial cells) but also adaptive changes such as the proliferation of chloride cells have been reported (Baker, 1969; Eisler and Gardner, 1973; Schreck and Lorz, 1978).

Contrary to the branchial epithelium, the skin surface has received very little attention, although this tissue is also in intimate contact with the environment and actually responds rapidly to pollutants such as cadmium or lead (Iger, 1992). The epidermis of teleost fish is a metabolically very active border tissue, consisting of several cell types including filament cells, mucous cells and sensory cells. In the skin of many species also chloride cells or club cells may be present (Whitear, 1986). The epidermis is covered by a mucus layer that forms an additional barrier to the water. The mucus contains protease, phosphatase, and peroxidase activities (Brown et al., 1990; Iger and Abraham, 1990; Iger and Wendelaar Bonga, 1994), has ion-concentrating capacities (Fromm, 1980), and may limit the penetration of heavy metals such as cadmium and mercury into the body, mainly due to its glycoprotein content (Pärt and Lock, 1983). The skin and its mucal secretion accumulate copper even during short episodes of exposure (Handy, 1992). However, there is a lack of information about the structural changes of this tissue during copper exposure.

In this paper ultrastructural changes are described in the epidermis and dermis of carp exposed for different periods to 100 μg l⁻¹ copper in the water, an environmentally relevant concentration (Spry et al., 1981; Laurén and McDonald, 1985). Our preliminary results (Lock et al., unpublished) indicate that copper exposure initiates significant elevation of plasma cortisol levels after 24 h. This elevation is considered to be a non-specific response to stressors. Therefore our results are also discussed in relation to the general cellular response of the skin to stressors.

2. Materials and Methods

Sixty-four juvenile (weighing 5–8 g) male and female carp, *Cyprinus carpio*, obtained from our laboratory stock, were kept in two groups for an acclimation period of...
3 weeks. Fish were maintained in aquaria filled with tap water (composition of the main ions in mmol l\(^{-1}\): 3.8 Na\(^+\); 0.06 K\(^+\); 0.8 Ca\(^{2+}\); 0.2 Mg\(^{2+}\); 5.5 Cl\(^-\); 0.3 HCO\(_3^-\) at pH 7.5 and 22°C. One group served as control. The other group was exposed to 1.6 \(\mu\)mol l\(^{-1}\) (100 \(\mu\)g l\(^{-1}\)) Cu. To reduce handling stress, each of the above mentioned groups was equally subdivided into sampling groups that were kept in separate aquaria. The desired copper concentration in the experimental aquaria was obtained by continually mixing the inflowing tap water with the appropriate amount of stock solution of Cu(NO\(_3\))\(_2\) by means of a multi-channel pump. The planned concentration was achieved 6 h after the start of the experiment. The actual copper concentration in the water was daily measured by atomic absorption spectrophotometry (PU 9200X, Philips), and was adjusted if the deviation was more than 10% from the planned concentration. The water of both groups was well aerated, and filtered, and continually refreshed with a daily replacement of 20% of the water.

For electron microscopy, we sampled 30 of the above-mentioned fish. Skin biopsies (about 3 \(\times\) 3 mm) from three fish of each group were taken 24 h, and 7, 14, 21 and 43 days after reaching the final concentration of copper. Samples were taken from the dorsal parts of the head of fish lightly anesthetized in neutralized MS 222. The tissues were fixed in 3% glutaraldehyde in sodium cacodylate buffer (0.09 M; pH 7.35), washed in this buffer and post-fixed in 1% osmium tetroxide in the same buffer. Ethanol dehydrated tissues were embedded in Spurr’s resin. Thin sections were contrasted with uranyl acetate and lead citrate and were examined in a Jeol 100 CXII transmission electron microscope. The detection of peroxidase activity was carried out on glutaraldehyde fixed skin samples of fish exposed to copper for 24 h and 7 days and with the conventional diaminobenzidine technique (see Iger and Abraham, 1990). Control specimens were incubated in the absence of H\(_2\)O\(_2\).

Epidermal thickness was measured in semi-thin (1.5 \(\mu\)m) sections. Data represent the mean ± sd of 7 to 10 sections per fish (three fish per group), with 40–50 \(\mu\)m intervals between the sections. The results were tested for statistical significance with the Student \(t\)-test.

3. Results

3.1. Control

Epidermis

The general ultrastructure of the skin of our carp has recently been described (Iger and Wendelaar Bonga, 1994). Briefly, the epidermis was 95–100 \(\mu\)m thick and was composed of several layers of filament-containing epithelial cells (filament cells) as well as mucous cells and club cells. Merkel cells and lymphocytes were occasionally observed. Chloride cells were not found.

Filament cells. The filament cells were interconnected by desmosomes and cytoplasmic interdigitations. Tight junctions were present between the cells of the outermost layer of filament cells, called the pavement cells. The apical membrane of the
pavement cells was folded into microridges. Their cytoplasm contained some electron-lucent vesicles. Occasionally the latter were seen to fuse with the apical cell membrane and to release their contents by exocytosis. Apoptotic pavement cells were hardly found. We did not observe mitotic filament cells, and hardly observed any necrotic or apoptotic pavement cells. The basal layer of filament cells was attached to the underlying basal lamina and adjacent connective tissue by hemidesmosomes. The filament cells displayed intense endocytotic activity at the basal part of the outer cell membrane.

Mucous cells. Newly differentiating mucous cells were found in the second and third inner layers of the epidermis. Such cells contained a centrally located nucleus surrounded by mitochondria, an extensive rER, and several Golgi systems from which small vesicles were budded. The latter coalesced to form oval electron-lucent mucosomes. Mature mucous cells, mostly goblet shaped, lost their desmosomes, and migrated towards the epidermal surface. Before discharging their content, the cells were 13–16 μm long. After discharge, the cytoplasmic remnants of the mucous cell were shed without showing signs of apoptosis. Endogenous peroxidase activity was hardly found in mucous cells.

Club cells. Mature club cells were located only in the middle of the epidermis. They contained a centrally located multilobed nucleus surrounded by a thin rim of central cytoplasm. Most of the cytoplasm was electron transparent and occupied by fine curved microfilaments, with a diameter of 4–5 nm. Newly differentiating club cells were seen in the second and third inner layers of the epidermis. Such cells were characterised by a centrally located nucleus surrounded by an extensive rER, free ribosomes and several Golgi systems. At the cell periphery a thin rim of helical microfilaments was present.

Merkel cells. Merkel cells were occasionally found in the 3 to 4 outermost layers of the epidermis. They were rounded, with 1 to 3 cytoplasmic extensions per cell, and contained 10 to 15 dense-cored secretory granules per cell section.

Dermis

The dermis was composed of collagen fibres, which appeared to be randomly arranged in the outer dermis, and highly compressed in the inner dermis. The main cellular elements in the inner dermis were fibroblasts. Some capillaries were also present. Their endothelial cells were actively engaged in cytosis. In the outer dermis fibroblasts were less frequent and capillaries were rare. Pigment cells, melanocytes, xanthocytes and iridocytes, were scarce in the inner dermis but were common close to the basal lamina. Melanosomes were located in the cell bodies of melanocytes and only rarely seen in the cytoplasmic extensions of these cells. Lymphocytes and basophilic granulocytes were hardly found. Other leucocytes were absent. Dermal papillae, containing axons and capillaries, were rare.
3.2. Copper contamination

**Epidermis**

After 24 h and 7 days of copper exposure the epidermal thickness decreased significantly from 98.1 ± 5.5 \( \mu\)m in the controls, to 89.2 ± 7.3 \( \mu\)m and 83.1 ± 6.75 \( \mu\)m \((P < 0.04 \text{ and } P < 0.002, \text{ respectively})\). During this period the skin surface was highly wavy. Later, the epidermal thickness gradually returned to control values of 102.8 ± 12.6 \( \mu\)m, 103.7 ± 16.1 \( \mu\)m and 86.7 ± 20.5 \( \mu\)m after 14, 21 and 43 days, respectively. The basal lamina appeared normal after 24 h, but became highly undulating later on.

**Filament cells.** Filament cells from the 4 to 5 outermost layers of the epidermis contained well-developed rER and Golgi systems. These cells were actively synthesising small secretory granules of high electron density (Fig. 1). At the end of the experiment (day 43) the granules were restricted to the pavement cells. The skin surface was covered by a pronounced glycocalyx (Fig. 2). The tight junctions between the pavement cells were intact during the experiment.

Mitotic filament cells, not observed in control fish, were frequently seen after 7 and 14 days. Mitosis occurred throughout the epidermis, except for the uppermost layer (the pavement cells) and the innermost layer of this tissue. Most mitotic cells were found adjacent to the club cells.

Necrotic pavement cells (swollen cells showing disrupted membranes, increased electron transparency of the cytoplasm and fragmentation of nuclear heterochromatin; Fig. 3) were frequently observed during the whole period. Apoptotic filament cells (shrunken cells showing condensation of cellular elements, increased electron density of cytoplasm, and loss of junctional complexes) were common during the first 14 days. Apoptosis occurred in pavement cells (Fig. 4) and also in some inner filament cells that were located adjacent to club cells (Fig. 5). Remnants of degenerated cells were occasionally found in phagosomes in the filament cells (Fig. 6).

**Mucous cells.** Most mucous cells, elongated rather than goblet shaped, were located in the outermost layers of the epidermis, and large quantities of amorphous mucus were seen on the skin surface (Fig. 7). From day 7 onwards, differentiating and newly differentiated mucous cells appeared. Most of them were located in the outer epidermal layers, occasionally adjacent to the pavement cells, i.e. one layer off the surface. After 21 and 43 days differentiating mucous cells were also found at their normal location, i.e. close to the basal filament cells.

During the first 21 days, the mucous cells contained abundant rER and mucosomes of high electron density (Fig. 9). Some mucosomes showed a core of high electron density, surrounded by a rim of mucus of moderate electron density. Other mucosomes (Fig. 8) had a transparent core and an electron dense periphery. At day 43, mucosomes of normal electron transparency were common. The mucosomes, having a diameter of about 0.5 \( \mu\)m, were smaller than in the controls and showed a positive reaction in the peroxidase test (Fig. 10).

Degenerating mucous cells, both newly differentiated and mature cells that had not
yet released their content, were seen after 21 and 43 days. We found both apoptotic and necrotic mucous cells. However, we could also find rounded mucocytes (Fig. 11) that contained a centrally located large membrane-bound vacuole of low electron
Fig. 7. A thick mucus layer, homogeneously spread on the skin surface; mc, mucous cell; 14 days of copper exposure; × 1800. Fig. 8. Mucous cell showing normal as well as highly electron dense mucosomes. In both mucosome types, electron transparent cores (arrows) can be found; 24 h of copper exposure; × 7200. Fig. 9. Mucous cell filled with mucosomes of high electron density; arrow, skin surface; 7 days of copper exposure; × 5600. Fig. 10. Peroxidase activity in mucous cell; arrow, skin surface; DAB reaction, otherwise unstained; 24 h of copper exposure; × 4400. Fig. 11. 'Clear' mucous cell, in mid-epidermis; arrow, remnants of mucosomes; 7 days of copper exposure; × 3000. Fig. 12. Leucocytes (l) present in the interior of a club cell (cc); fc, filament cell; 24 h of copper exposure; × 3500.

density. Occasionally remnants of membranes of mucosomes were present in the vacuole. Also mucous cells with fused mucosomes were found. These cells were goblet-shaped and contained several fused mucosomes at their apical pole.
Club cells. Club cells were elongated with their long axis perpendicular to the epidermal surface. The cells were located close to the skin surface. They appeared very active: the cytoplasm around the nucleus contained extensive rER, Golgi systems, and many free ribosomes. Small vesicles, probably lysosomes, were found adjacent to the Golgi area and close to the outer cell membrane. Club cells containing large phagosomes were frequent during the first 14 days. After this time, many newly differentiated club cells were seen at their normal location but also closer to the skin surface.

Chloride cells. After 7 and 14 days, single chloride cells and, occasionally, clusters of 2 to 3 of these cells, appeared in the outer cell layers. The chloride cells had the typical components of this cell type, i.e., an extensive tubular system, many mitochondria and a small apical crypt. At their apical poles, chloride cells were connected by desmosomes and tight junctions to adjacent pavement cells. Occasionally necrotic- and apoptotic chloride cells (Wendelaar Bonga and Van der Meij, 1989) were seen. Some of the apoptotic cells were engulfed by macrophages.

Merkel cells. During the first 7 days Merkel cells were depleted of their secretory granules (Fig. 14). During this period also necrotic (Fig. 13) and apoptotic Merkel cells were found. Later on normal Merkel cells, each with 5 to 10 granules per cell section, were more common than in the controls.

Leucocytes. During copper exposure, many leucocytes extravasated and penetrated the epidermis. After 24 h many lymphocytes (Fig. 15) and basophilic granulocytes were found, mainly in the inner layers of the epidermis. Later on, also other types of leucocytes, i.e. eosinophilic- (Fig. 16) and neutrophilic granulocytes, macrophages (Fig. 17), and plasma cells, were seen in the dermis and throughout the epidermis. The number of leucocytes (an estimation based on electron microscope observations) varied from 320 to 400 cells per 1 mm length of epidermis during the whole experiment. Leucocytes, in particular macrophages and basophils, seemed activated: they contained well developed rER, many Golgi systems, lysosome-like vesicles and several phagosomes. The latter appeared mainly during the first 14 days, and after 43 days. Necrotic and apoptotic basophilic granulocytes as well as mast cells were common. Remnants of such degenerated cells were detected within macrophages. Several lymphocytes and granulocytes were found inside club cells (Fig. 12).

Dermis

Fibroblasts. Many fibroblasts were located near the basal lamina. They were very active, as was reflected by the presence of abundant rER, several Golgi systems and small peripheral vesicles in the fibroblasts, and by the large amounts of collagen secreted by these cells. The outer dermis became filled with collagen fibres from day 7 onwards.

Capillaries. The pinocytotic activity of the endothelial cells diminished after 24 h but was restored later. At day 7, many endothelial cells were elongated in the direction of
the basal lamina. Later on capillaries were common also in the outer dermis, indicating angiogenesis during copper exposure.

**Pigment cells.** During the first 14 days, the skin of the copper exposed fish was
darker than that of the controls. During this period pigment granules were distributed in the cytoplasmic extensions of the melanocytes rather than in the cell bodies. Many of these extensions were close to the basal lamina, but did not penetrate the epidermis. Occasionally apoptotic remnants of melanocyte extensions were found, mainly within macrophages. After 21 and 43 days, the granules became more and more confined to the cell bodies of the melanocytes, as in the controls. In these samples we found many axons adjacent to the melanocytes. Usually we found melanocytes with pseudopodial-like cytoplasmic processes surrounding these axons (Fig. 18).

4. Discussion

The results of this study show that exposure of fish to 1.6 μmol l⁻¹ of copper has marked and prolonged effects on the epidermal and dermal skin layers. Most of the morphological changes represent adaptive responses of the skin. However, part of the changes might be considered as direct effects of copper, mostly of a degenerative nature (i.e. necrosis of pavement cells). A complete recovery of this tissue was not found during the 43 days of the exposure period.

Epidermis

We found that the thickness of the epidermis was reduced during the first 7 days of copper exposure. Changes in epithelial thickness have commonly been described in fish exposed to environmental stressors, e.g. for the epidermis of winter flounder exposed to crude petroleum (Burton et al., 1984), the epidermis of carp exposed to manure, lead or acid water (Iger et al., 1988; Iger, 1992; Iger and Wendelaar Bonga, 1994) or the gill epithelium of tilapia exposed to acid water (Wendelaar Bonga et al., 1990). The decrease in epidermal thickness may reflect the damage to the upper cell layers caused by copper, while the subsequent increase in thickness may be considered as an adaptive response (see below).

Filament cells. The decrease in epidermal thickness and the wavy appearance of the skin surface during the first 7 days were associated with degeneration, by necrosis and apoptosis, and shedding of pavement cells at the epidermal surface. Necrotic processes reflect accidental cell death (Wyllie, 1981), and thus likely represent direct toxic effects of the copper on the superficial cells. Copper was also shown to induce degeneration of the upper cell layers of the skin of the brown bullhead and of the branchial epithelial cells of the catfish (Benedetti et al., 1989; Khangarot and Tripathi, 1991). Necrosis of pavement cells occurred also after exposure to zinc or cadmium (Somasundaram, 1985; Iger, 1992) as well as after exposure to low pH water (Wendelaar Bonga et al., 1990).

The increase of apoptosis, defined as physiologically controlled cell death (Wyllie, 1981) may have been triggered by slight, non-lethal, damage to the cells concerned or reflects reduction of the normal life-span of the cells as a result of stimulated cellular activity. The increased synthesis and secretion of glycocalyx by the upper layers of filament cells may have caused premature ageing and exhaustion. The high activity of
the external filament cells, the apoptosis, and the mitosis of inner filament cells indicate that the turnover of the filament cells increased markedly during copper exposure. Apoptosis was also found in skin of carp exposed to cadmium (Iger, 1992) and acid water (Iger and Wendelaar Bonga, 1994), and in the gill epithelium of acid-exposed tilapia (Wendelaar Bonga et al., 1990). Phagocytosis of apoptotic cells or cellular remnants by filament cells, as reported here, have previously been reported for carp and rainbow trout skin (Iger and Abraham, 1990; Peleteiro and Richards, 1990). The regulation of epidermal cell proliferation in fish is influenced by cortisol as well as prolactin (Wendelaar Bonga and Meis, 1981; Iger, 1992). Exposure of coho salmon to copper produced a marked elevation in serum cortisol (Schreck and Lorz, 1978). It is possible that the elevated cortisol levels observed in our copper exposed carp (Lock et al., 1992) initiated the filament cell proliferation.

The penetration of the epidermis by many leucocytes may have contributed to the epidermal thickening. During this phase of the experiment, the overall increase in cell mass apparently surpassed the loss by cell shedding. Another factor frequently contributing to increased epidermal thickness, the enlargement of the intercellular spaces (a phenomenon widely observed in epithelium of stressed fish; Whitear, 1986; Wendelaar Bonga and Lock, 1992), did not occur in our experiments.

**Mucous cells.** Mucus secretion was stimulated by copper. This has been reported earlier for copper (Benedetti et al., 1989; Khangarot and Tripathi, 1991) as well as for many other pollutants, e.g. crude petroleum, heavy metals such as lead or mercury, or water acidification (Burton et al., 1984; Iger, 1992; Lock and Van Overbeeke, 1981; Wendelaar Bonga et al., 1990). The appearance of a thick mucus layer attached to the epidermal surface may be caused by the coagulation of mucus under the influence of the copper ions (Khangarot and Tripathi, 1991). At least two other, possibly interrelated, phenomena, that were both observed in our experiment, have been associated with increased viscosity of the mucus: a smaller size of the mucosomes (Lewis, 1976), and changes in mucous composition (Gona, 1979).

Changes in chemical composition of the mucus were indicated in our experiment by the increased electron density of the mucosomes during copper exposure. Cells containing dense mucosomes have been termed serous mucous cells (Whitear, 1986). Electron-dense mucosomes in the mucous cells of trout contain a basic proteinaceous material (Blackstock and Pickering, 1980). In our carp the appearance of electron-dense mucosomes was associated with the appearance of peroxidase activity, as we have shown recently for carp from acid water (Iger and Wendelaar Bonga, 1994). This enzyme may contribute to the antibacterial properties of the mucus. Whether the changed mucous composition effects its ion-binding capacities remains to be established. Changes in Na⁺ concentration have been noticed in the mucus of carp exposed to several stressors (Lebedeva et al., 1989). Copper can disrupt transepithelial ion exchange and thus alter the Na⁺ concentration in the blood plasma (Reid and McDonald, 1988).

In mucous cells of the brown bullhead exposed to 0.3 mg l⁻¹ copper, changes in mucus composition were observed by PAS and Alcian Blue staining (Benedetti et al., 1989). These authors presumed that the changes were associated with the presence of
newly differentiated mucous cells, more closely to the skin surface. Our results confirm that differentiation of mucous cells in fish exposed to copper occurs closer to skin surface, than in control fish. Similar changes in mucus composition and in location of mucous cell differentiation were also found in carp exposed to water polluted with manure (Iger et al., 1988).

**Club cells.** The migration of the club cells towards the surface of the epidermis, during exposure to copper, may facilitate the release of their alarm substance (Pfeiffer, 1977). We also observed migration of club cells after exposure of carp to cadmium, lead, manure, brackish water or low water pH (Iger et al., 1994). As we have discussed recently, the location of mitotic and inner apoptotic filament cells adjacent to club cells suggests that club cells are involved in the epidermal cellular regulation, in addition to the secretion of alarm substance (Iger et al., 1994). Club cells contain serotonin (Fujita et al., 1988; Zaccone et al., 1990) as well as crinotoxins (Al Hassan et al., 1987). The apparent penetration and degeneration of leucocytes into club cells as found during copper exposure was also observed for the other stressors mentioned (Iger et al., 1994). The question was raised whether these substances play a role in the elimination of penetrated leucocytes. The present results support our earlier conclusion that club cells may have multiple functions.

**Chloride cells.** In control carp, chloride cells are present only in the gills and in the epithelium covering the inner side of the operculum (Wendelaar Bonga, unpublished). These cells have an important function in ion regulation. Their appearance in the skin may be associated with the disturbing effects of copper on ion exchange (in particular Na⁺-influx) in the branchial epithelium (Reid and McDonald, 1988). The appearance of chloride cells in the skin after 7 and 14 days is consistent with the period of disturbance in the total branchial transport capacity of trout exposed to 55 μg l⁻¹ copper and may be a response to this disturbance (Lauren and McDonald, 1987). Their disappearance from the skin at the end of the experimental period may reflect that the ionic regulation has been restored, for instance by adaptive processes in the gill e.g., an increase in chloride cell number in the branchial epithelium. Such an increase has been reported for the winter flounder and the dogfish after copper exposure (Baker, 1969; Crespo et al, 1981). The appearance of chloride cells in the skin of carp can be initiated by administration of ACTH, likely via cortisol (Iger, 1992). Cortisol stimulates chloride cell proliferation in the gills (McCormick, 1990). Since cortisol is released in response to a wide range of stressors, including copper, we suggest that the initial appearance of chloride cells in the skin of copper-exposed carp is a general response to a stressor, rather than a specific response to copper.

**Merkel cells.** The appearance of necrotic Merkel cells, an observation that has not been reported before, suggests a direct effect of copper on these cells. Degeneration of sensory elements, mainly taste buds, has been reported for fish exposed to copper (Benedetti et al., 1989), mercury (Pevzner et al., 1986), and cadmium (Iger, 1992). The observed increase in the number of Merkel cells at the end of the experiment might be a response to compensate the thick and viscous mucous layer on the epidermis that
may hampering their sensory function, as has been suggested for *Alburnus alburnus* exposed to mercury (Pevzner et al., 1986).

**Leucocytes.** Copper exposure evoked infiltration of the epidermis by many leucocytes. We have previously described leucocyte infiltration in the skin of carp exposed to manure (Iger et al., 1988), lead (Iger 1992), or acid water (Iger and Wendelaar Bonga, 1994) as well as after wounding of the skin (Iger and Abraham, 1990). Massive migration of leucocytes has also been described for the branchial epithelium under the impact of stressors including many water pollutants (Mallatt, 1985; Karlsson-Norrgren et al., 1985; Wendelaar Bonga and Lock, 1992). The migration of leucocytes into these epithelia can at least partially explain the leucopenia that is characteristic for fish exposed to stressors (Pickering and Pottinger, 1987; Maule and Schreck, 1990), including copper (Khangarot and Tripathi, 1991).

Basophilic granulocytes were among the first to appear in carp epidermis under the impact of copper. This is consistent with our earlier observations on carp after wounding or exposure to lead and manure (Iger et al., 1988; Iger and Abraham, 1990; Iger, 1992). These cells were absent in the epidermis of trout exposed to acidified water (Iger and Wendelaar Bonga, 1994), temperature elevation or chlorine pollution (Iger and Wendelaar Bonga, unpublished). Their rapid appearance in carp epidermis and their intense phagocytic activity are probably connected with their functioning as major components of the cellular immune response. The high activity of these cells is also reflected by the appearance of apoptotic basophils. Also mast cells exhibited signs of apoptosis or necrosis. Necrotic mast cells were reported earlier for the gills of *Mytus bleekeri* exposed to copper (Gupta and Kajbanshi, 1981). Degenerated basophilic granulocytes and mast cells have been reported in fish exposed to lead (Iger, 1992).

**Dermis**

**Fibroblasts.** We have previously reported (Iger and Abraham, 1990) that the appearance of many fibroblasts in the outer dermal zones is caused by their migration to these areas, rather than by mitotic activity. The increased synthesis and secretory activity of fibroblasts occur also in carp exposed to cadmium or lead (Iger, 1992) or after wounding (Iger and Abraham, 1990), as well as in flatfish exposed to water contaminated with sewage sludge (Bucke et al., 1983). In carp, this activity can be stimulated by injection of ACTH (Iger, 1992). This activity, that results in a highly fibrous dermis, may reflect a general response to stressors. The accumulation of fibroblasts under the skin epithelium may further be associated with their ability to synthesize metallothioneins after copper exposure (George et al., 1992). Metallothioneins play a pivotal role in the adaptation to heavy metals, including copper (Dixon and Sprague, 1981).

**Capillaries.** The primary responses observed in the endothelial cells were the reduction of their pinocytotic activity and angiogenesis. Similar responses have been detected in the skin of carp after wounding (Iger and Abraham, 1990), after exposure to lead (Iger, 1992) or in the oesophageal epithelium of *Oreochromis mossambicus* dur-
ing adaptation to different salinities (Cataldi et al., 1988). Angiogenesis may be associated with the high nutritional requirements caused by increased cellular activity and turnover rate of the epidermis under the influence of stressors.

**Pigment cells.** The increased pigmentation observed in the skin of copper exposed fish was caused by dispersion of pigment granules in the cytoplasmic extensions of melanocytes. This was also reported for carp skin after contamination with manure or lead (Iger, 1992). In contrast to this study, in our present experiment on copper, melanocyte extensions were not detected within the epidermis. After 21 and 43 days most pigment granules were re-aggregated in the cell bodies of melanocytes. Re-aggregation of pigment granules is effected by adrenergic neurons (Schliwa, 1986). In our study, the reaggregation of pigment was associated with the appearance of axons, perhaps adrenergic ones, adjacent to melanocytes.

In conclusion, the wide range of pronounced changes observed in the skin of carp exposed to copper, demonstrate the sensitivity of the tissue to this metal. The responses observed in the skin seem to be part of a general response to stressors, rather than a specific response to copper.

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**References**


