In conclusion, our data indicate that *L. donovani* infects and survives in lymphocyte cell lines *in vitro*. Many of the immunopathological responses in visceral leishmaniasis have traditionally been attributed to infection of macrophages. Our present findings raise the possibility that lymphocytes may be infected *in vivo* contributing to their observed functional impairment in visceral leishmaniasis. This possibility merits careful study with patients. Production of IL-12 by B cell line in response to *L. donovani* suggests that B cells may play a key role in the early phase of *Leishmania* infection.

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Estimation of sublethal toxicity of zinc chloride by histopathological analysis of fish (*Heteropneustes fossilis*, Bloch) epidermis

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The effect of zinc chloride on the outer (OE) and inner (IE) opercular epidermis of *Heteropneustes fossilis* has been investigated. The main toxicopathological alterations of the OE include extensive intercellular as well as intracellular vacuolization and hyperplasia of the epithelial cells (ECs) with regular exfoliation of round or globular ECs from the skin surface. Splitting of the epidermis from the junction of the outermost (OL) and middle layers (ML) leading to the lifting of the OL is sometimes also noticed. The mucous cells (MCs) show periodic fluctuations in their density and staining properties. Extensive vacuolization of the epidermis along with hyperplasia of ECs along with periodic fluctuations in the density and staining behaviour of the MCs are the main alterations observed in the IE. The mucogenic activity of the IE throughout the exposure period remains mostly above the control level. Hence the damage is comparatively less severe. All these histo-pathological manifestations may be considered for their use for testing the quality of variously contaminated water samples.

HEAVY metal pollution represents a threat to the aquatic biota. The occurrence of metal contaminants especially zinc in excess of natural loads has become a problem of increasing concern. This situation has arisen as a result of the rapid growth in population, increased urbanization, expansion of industrial activities, exploration and exploitation of natural resources as well as lack of environmental regulations. The gills have extensively been used as a potential indicator for disturbed aquatic environment. The other important organ system that also gets similar flooding is the skin. However, data dealing with the impact of various heavy metals including zinc salts are very scanty[1–6]. While studying the acute toxic impact of heavy metal salts on melanophore morphology, Banerjee and his associates[5,7] have demonstrated the importance of melanophore indexing in testing water qualities contaminated with lethal concentrations of mercury and zinc chloride. Application of similar melanophore indexing bio-assay technique, however, failed to evaluate the water samples polluted with sublethal concentration of heavy metal salts including zinc chloride. Hence in this paper efforts have been made to evaluate the effect of zinc chloride on

the skin of *H. fossilis* in an attempt to explore the possibility of the extensive use of the epidermis as a potential bioindicator.

Live specimens of *H. fossilis* weighing 35–40 g and measuring 18–20 cm were acclimated in large plastic aquaria for one month. Fish were fed with minced goat liver and the water was renewed every morning. For the study of sublethal toxicity, 5 groups of 10 fish each were exposed to 7.5 ppm (10% of 96 h LC_{50} value (= 75 ppm)[8] of zinc chloride (99% pure; E Merck India Ltd., Mumbai). The test solution was prepared in 50 l of tap water (having dissolved oxygen 6 mg/l, pH 7.5, water hardness 23.2 mg/l and water temperature 24 ± 2°C). In similar parallel control groups, zinc salt was not added. Feeding was allowed for a period of 3 h every day, just before the renewal of the media. Opercula from both the sides of the fish were fixed in aqueous Bouin's fluid, 10% neutral formalin and Helly's fluid after the expiry of each of the 5, 10, 20, 30 and 45 days of exposure period. Six μm vertical sections (VS) was stained in Ehrlich's haematoxylin/eosin (H/E) for histopathological analysis. Glycoproteins were histochemically detected by periodic acid-Schiff (PAS), alcian blue pH 2.5 (AB 2.5) and AB 2.5/PAS methods[9]. While sulphated mucosubstances were localized by alcian blue pH 1.0 (AB 1.0) method, water stable mucoproteins were detected by the Bismarck brown (BB) technique[9]. Glycogen was visualized by PAS, salivary amylase/PAS techniques[9]. The thickness of both the epidermal linings was calculated using ocular and stage micrometers. The density of the epithelial cells (ECs) of the outer as well as inner epidermal linings was measured from VS of the operculum. One way analysis of variance (ANOVA) following Duncan's multiple range tests was also applied to detect if the data related to the thickness and density of ECs are significantly affected by exposure periods (Figures 1 *i, j*).

The main cellular elements of the outer opercular lining are ECs, club cells (CCs) and goblet mucous cells (MCs) (Figures 1 *a, b*). The MCs are usually found in the outer layer. The middle portion of the epidermis is characterized by the presence of a single layer of large sized CCs whose contents often appear very finely granulated. ECs usually fill the interstices between the gland cells. The thin inner opercular lining differs from the outer one in not possessing any CC. Tables 1 and 2 give histochemical properties of the various cellular components of the outer and inner opercular epidermis.

The sublethal toxicity of zinc chloride on the skin of *H. fossilis* is slowly manifested hence not very prominently exhibited after 5 days of treatment. Although the number of the MCs increases marginally, they reduce in size acquiring horizontally elliptical shape (Figure 1 *d*). Space thus vacated by the MCs (due to their reduced dimension) gets promptly filled with the closely aggregated rectangle ECs which remain separated from their neighbours by prominently visible intercellular spaces. The CCs at this stage acquire cuboidal shape and at certain locations they appear smaller. Few EGCs which in the control fish are not generally located, are sometimes observed at this stage (Figure 1 *c*). Subsequently, the ECs in the outermost layer acquire round or polygonal shape, remaining closely approximated giving this layer a compact appearance. Exfoliation of ECs individually or in small flakes is regularly observed after 10 days of exposure. The ECs between the two MCs also appear less vertically compressed. The MCs take sac-like shape (mostly in the outermost layer) after 10 days of exposure when their number increases greatly due to regeneration of new MCs in the ML. Often a second layer of MCs is also observed in the outermost layer. These MCs stain variously with the AB 2.5/PAS (Table 1) method with the different segments of the same MCs very often staining differently with this technique. Several tiers of ECs, lying just over the CCs acquire spindle shape and become horizontally flattened. This layer appears prominently vacuolated. The cell junctions between the neighbouring ECs loosen to cause increase in the intercellular spaces with consequent wear and tear of the most superficial layer after 20 days of exposure. However, the number of MCs that continues to rise, reaches its highest level at this stage of exposure. The height of the MCs also increases during this period when the bottom of the MCs extends quite deep. Fine intercellular vacuoles are commonly noticed in the cytoplasm of many of the ECs which also exhibit oedematous swelling. The space vacated by the degenerated gland cells also gets quickly occupied by the rapidly multiplying ECs, resulting in an altered morphology and thickness of the epidermis (Figure 2). A thick layer of amorphous eosinophilic substance often covers the surface of the epidermis after 30 days of exposure (Figure 1 *g*) when the number and height of the MCs decrease significantly (number, however, still remains marginally above control level). The size of the MCs also increases significantly whose lateral pressure compresses the ECs vertically. This renders the outermost layer of the epidermis the control-like configuration. The decrease in the number of the MCs continues when it becomes subnormal after 45 days. At this stage, the epidermis appears loosely arranged specially at its outermost layer, with individual ECs at the surface layer being detached from their neighbours. Before exfoliation, these cells acquire round, globular dimension. After 5 days of exposure, the size of some of the CCs decreases. These cells acquire cuboidal shape with their contents covering their entire space. Vacuolization in the perinuclear areas of these cells increases which, however, decreases after 10 days (Figure 1 *e*). After 30 days, two tiers of CCs are frequently observed. However, the density of CCs

decreases after 45 days. Vacuolization also appears in the lower layers of the epidermis following exposure. At the later part of the experiment, wear and tear of the ECs takes place, resulting in appearance of large vacuole-like spaces between neighbouring ECs. Like their density the MCs also exhibit periodic alteration in their staining properties (Table 1). The staining behaviour of the ECs of the most superficial layer, as well as the slimy coatings over the epidermis also show periodic fluctuations (Table 1). The thickness/quantity of the slimy coating over the body surface also fluctuates at different stages of exposure.

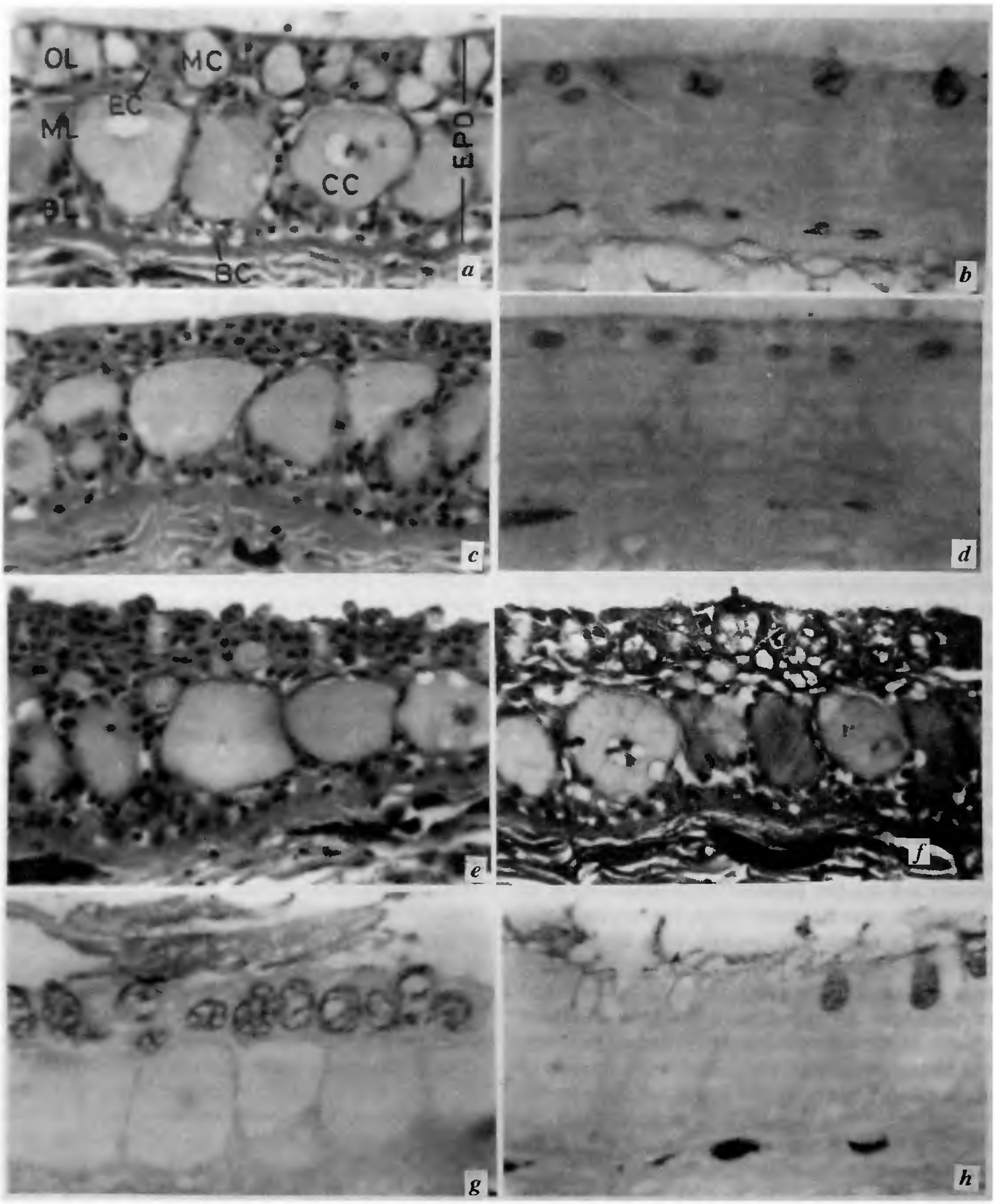

Figure 1 *a–h*.

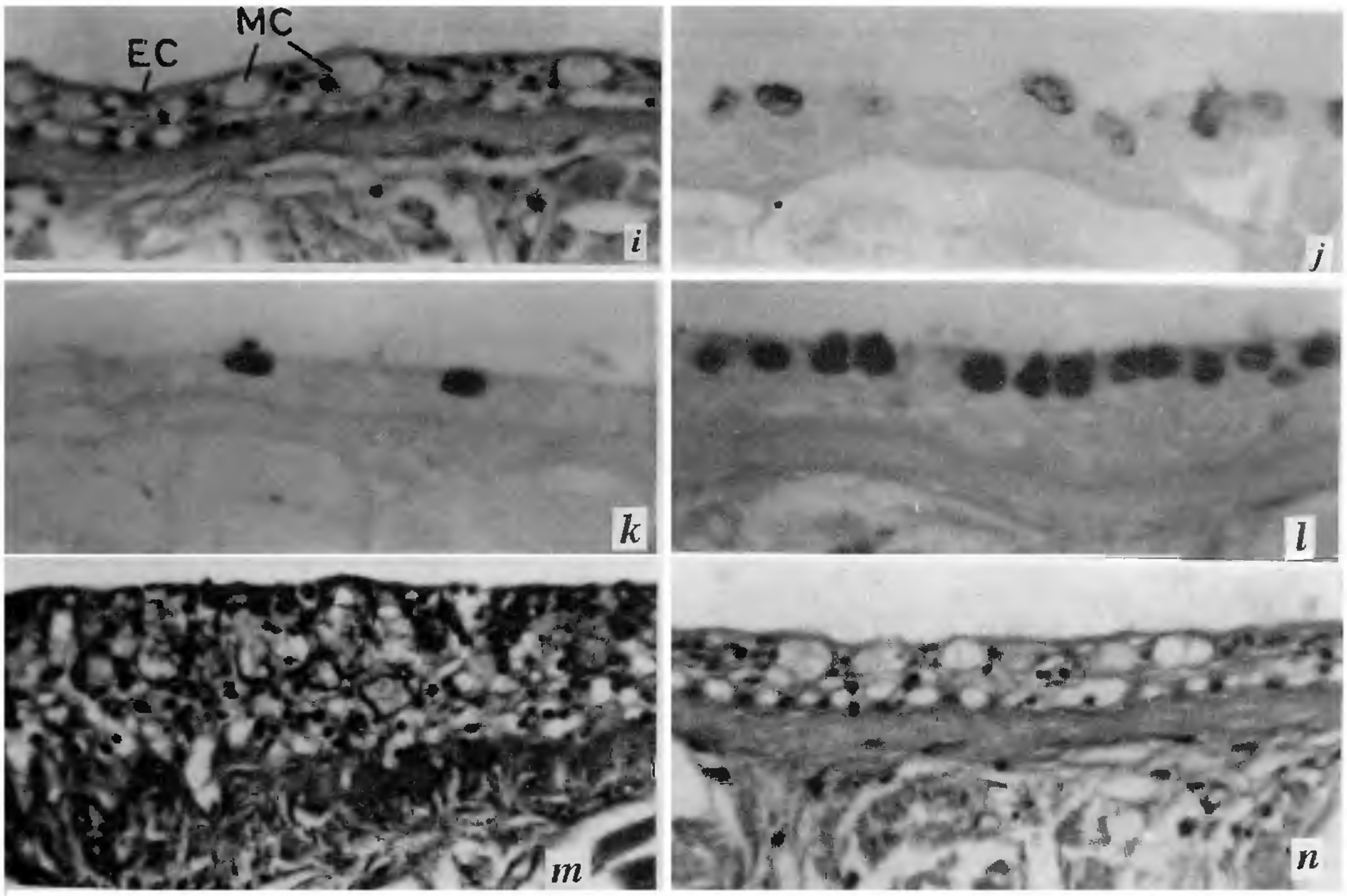

Figure 1 *a–n*. *a*, Vertical section (VS) of the operculum of the control fish showing the structural organization of its outer epidermal lining. (H/E, ×475). (BC, Basal cell; BL, Basal layer; CC, Club cell; EC, Epithelial/epidermal cell; EPD, Epidermis; MC, Mucous cell; ML, Middle layer; OL, Outermost layer). *b*, VS of outer epidermal lining of the operculum of control fish showing the normal distribution of carbohydrates especially in its MCs. (AB 2.5/PAS, ×475). *c*, Appearance of eosinophilic granular cell after 5 days of exposure (arrows) (H/E, ×475). *d*, Decrease in dimension of the MCs after 5 days of exposure (AB 2.5, ×475). *e*, Exfoliation of isolated ECs at the surface of the epidermis after 10 days of exposure (H/E, ×475). *f*, Few wandering cells (leukocytes) penetrating a CC after 20 days of exposure (H/E, ×475). *g*, Laying down of a thick coating of slime over the skin surface resulting in decreased density of MCs (from that the previous stage) after 30 days of exposure (AB 2.5, ×475). *h*, Further decrease in the density of the MCs after 45 days. Note the decreased quantity of slime over the skin surface perhaps due to sloughing (AB 2.5, ×475). *h*, VS of the inner epidermal linings of the operculum of control fish showing its structural organization (H/E, ×475). (EC, Epithelial Cell; MC, Mucous Cell). *j*, VS of inner epidermal lining of the operculum of control fish showing the normal distribution of carbohydrate moieties especially in its MCs. (AB 2.5/PAS, ×475). *k*, Decreased density of MCs after 5 days of exposure (AB 2.5/PAS, ×475). *l*, Hyperplasia of MCs after 10 days of exposure (AB 2.5/PAS, ×475). *m*, Hyperplasia of the epidermis causing its increased thickness after 30 days of exposure (H/E, ×475). *n*, Decrease in HEC resulting in the thinning of the epidermis after 45 days of exposure. Note the persistence of vacuoles in the lower layers (H/E, ×475).

The MCs of the inner opercular lining also show periodic fluctuation in their density that decreases substantially in the initial stages of exposure (Figure 1 *k*). After 10 days, the number of MCs increases markedly (Figure 1 *l*) which although fluctuates periodically, always remains above the control level. The staining properties of the different MCs of the same or different stages of exposure also show periodic alterations (Table 2). Hyperplasia of the ECs (Figures 1 *m*, *n* and Figure 3) with prominent intercellular vacuoles (rendering spongy appearance to the epidermis) is the main histopathological manifestation noticed in most of the exposure periods. However, vacuolization decreases in the outer layers in the later part of the experiment when only the lower layers show vacuolization (Figure 1 *n*).

Profuse secretion of slime over the opercular surfaces by the goblet MCs is perhaps the first response of the skin to the zinc chloride toxicity because mucus provides a defence mechanism against toxic substances such as heavy metals[1–4,10] as the mucous coating on the fish body acts as ion binding resin due to the capability of the metals to form a covalent bond with SH-group of proteins, S-containing amino acids and wide ranges of biological molecules[11–17]. The secretory activity of ECs of the superficial layer of the opercular epidermis also shows (periodic) fluctuations at many stages of exposure. The mucoid secretion of these cells might also be eliminating some of the zinc salt after trapping the heavy metals which might be approaching towards these cells following the exposure. Continuation of treatment with zinc chloride, however, does not prevent regeneration of the MCs and after 20 days the density of the

Table 1. Summary of the histochemical alterations in the carbohydrate contents of the various cell types of outer opercular epidermis of *H. fossilis* at various intervals of exposure

Cell			Control	5 days	10 days	20 days	30 days	45 days
PAS technique for glycoproteins (1,2 glycols)								
OL	ECs		1	1 ~ 2	±	3	1 ~ 2	1
	MCs	Periphery	2 ~ 3	2 ~ 3	3 ~ 4	3 ~ 4	2 ~ 3	3 ~ 4
		Contents	2 ~ 3	2 ~ 3	3 ~ 4	3 ~ 4	2 ~ 3	3 ~ 4
		S	1 ~ 2	0	0	3	2 ~ 3	4
ML	MCs	Periphery	2 ~ 3	Ab	3 ~ 4	3 ~ 4	3 ~ 4	3 ~ 4
		Contents	2 ~ 3	Ab	3 ~ 4	3 ~ 4	3 ~ 4	3 ~ 4
AB 1.0 technique for sulphated mucopolysaccharides								
Negative reaction throughout the epidermis in all the stages								
AB 2.5 technique for sulphated mucopolysaccharides								
OL	ECs		± ~ 1	2	± ~ 1	0	0	±
	MCs	Periphery	2 ~ 3	2	2 ~ 3	1 ~ 2	1 ~ 2	2 ~ 3
		Contents	1 ~ 2	2	2 ~ 3	1 ~ 2	1 ~ 2	2 ~ 3
		S	3	0	2 ~ 3	2 ~ 3	± ~ 1	2 ~ 3
ML	MCs	Periphery	Ab	Ab	2 ~ 3	2 ~ 3	1 ~ 2	1
		Contents	Ab	Ab	2 ~ 3	2 ~ 3	1 ~ 2	1
AB 2.5/PAS technique for acidic and neutral glycoproteins								
OL	ECs		+ ~ 1G	2 ~ 3G	2 ~ 3B	0	0	1R
	MCs	Periphery	2 ~ 3G	3 ~ 4B	3 ~ 4VS	3VS	2VS	3VS
		Contents	2 ~ 3G	3 ~ 4G	3 ~ 4VS	3VS	2VS	3VS
		S	3G	3 ~ 4G	4G	4B	2 ~ 3VS	3 ~ 4VS
ML	MCs	Periphery	2 ~ 3G	4B	3 ~ 4VS	3 ~ 4B	2VS	3 ~ 4VS
			2 ~ 3G	4B	3 ~ 4VS	3 ~ 4B	2VS	3 ~ 4VS
Salivary amylase/PAS technique for glycogen								
Negative reaction throughout the epidermis in all the stages								
BB for water stable mucoproteins								
OL	ECs		± ~ 1	1 ~ 2	3 ~ 4	± ~ 1	± ~ 1	2 ~ 3
	MCs	Periphery	0	2 ~ 4	2 ~ 3	0	0	0
		Contents	0	2 ~ 4	1 ~ 2	0	0	0

Symbols and abbreviations: AB 1.0, alcian blue at pH 1.0; AB 2.5, alcian blue pH 2.5; PAS, Periodic acid Schiff; AB/PAS, alcian blue pH 2.5/Periodic acid Schiff; B, Blackish green; BB, Bismarck brown; EC(s), epithelial cell(s); G, greenish blue; BC, basal cell; BL, Basal layer; CC, Club cell; EC, Epithelial/epidermal cell; EPD, Epidermis; MC, Mucous cell; ML, Middle layer; OL, Outermost layer; MC(s), mucous cell(s); ML, middle layer; OL, outermost layer; R, red; S, secretory coating; VS, variously stained; 0, negative reaction; ±, faint/doubtful reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction; 4, very strong reaction; ~, to.

MCs reaches the highest degree surpassing greatly the density of the MCs of the control fish perhaps due to stimulatory effects of the zinc salt.

However, further continuation of exposure again causes increased density of the opercular mucocytes after 30 days. The pattern of the secretory activity of opercular goblet MCs following zinc chloride treatment is quite different from that of mercuric chloride (another heavy metal salt) exposure[18]. In mercury-exposed fish, the periodic alteration in the density of MCs is much faster than that of the zinc (where the periodic alterations are quite slower). This is perhaps due to severe toxic nature of mercury salt (in contrast to zinc salt which is an essential trace element). However, prolongation of zinc salt exposure results in collapsing of this first line barrier system offered by slime because the slime gets easily dissolved out into the medium subjecting the cellular elements of the epidermis itself to the toxic stress of the zinc salt. The MCs of both the epidermal linings (inner and outer) of the operculum of the exposed

Table 2. Summary of the histochemical alterations in the carbohydrate contents of the various cell type of inner opercular epidermis of *H. fossilis* at various intervals of sublethal zinc chloride exposure

Cell			Control	5 days	10 days	20 days	30 days	45 days
PAS technique for glycoproteins (1,2 glycols)								
OL	ECs		1 ~ 2	±	±	2 ~ 3	2	2
	MCs	Periphery	3	3 ~ 4	3 ~ 4	4	3	3 ~ 4
		Contents	3	3 ~ 4	3 ~ 4	4	2	3 ~ 4
		S	0	0	0	0	3	0
AB 2.5 technique for sulphated mucopolysaccharides								
OL	ECs		1	2 ~ 3	2	± ~ 1	0	± ~ 1
	MCs	Periphery	3	3	2	1 ~ 2	2 ~ 3	2 ~ 3
		Contents	3	3	2	1 ~ 2	2	2 ~ 3
		S	0	0	0	3	3	0
AB 2.5/PAS technique for acidic and neutral glycoproteins								
OL	ECs		0	2G	2G	2R	1R	1 ~ 2VS
	MCs	Periphery	2 ~ 3G	4G	4VS	3 ~ 4VS	2G	3 ~ 4VS
		Contents	2 ~ 3G	4G	4VS	3 ~ 4	2G	3 ~ 4VS
		S	0	0	0	3 ~ 4G	2R	0
Salivary amylase/PAS technique for glycogen								
Negative reaction throughout the epidermis in all the stages of exposure								
BB for water stable mucoproteins								
OL	ECs		±	0	1	±	±	± ~ 1
	MCs	Periphery	1 ~ 2	3 ~ 4	3 ~ 4	3 ~ 4	3 ~ 4	3
		Contents	1 ~ 2	3 ~ 4	3 ~ 4	3 ~ 4	3 ~ 4	3

Symbols and abbreviations are as in Table 1.

fish not only show quantitative alterations at different stages of zinc chloride exposure, they also exhibit qualitative alterations within the same or different stages of exposure (Tables 1, 2). The same or different MCs of the skin, at different stages of experimentation show varying intensities of PAS and/or AB 2.5 positive reactions, indicating synthesis of slime containing acidic or a mixture of neutral and acidic glycoproteins[19–24]. It was also observed that the MCs in the lower and middle layers generally show more strong PAS reaction. During their migration towards the outer surface, these cells progressively show increased alcianophilia, indicating a change in the nature of the mucus (from neutral to acidic and/or weakly sulphated). Similar alteration in staining property (from neutral to acidic mucosubstances) of the MCs of the opercular epidermis during their migration from middle to outermost layer have also been observed by Zuchelkowski *et al.*[23] and Paul[24] following exposure to acid waters and ammonium sulphate solution respectively. It is well illustrated that acute lethality of dissolved zinc is reduced at low pH. The shift in the nature of the mucus towards acidity and/or weak sulphation as revealed by increased AB 2.5 reaction is thus significant as the mucous film over the body surface perhaps reduces the acute toxicity of the zinc by providing a thin layer of water stable BB positive slimy coating of viscous nature. Also, the composition and distribution of mucus may be affected by physiochemical features of water[25–27]. The collapse of the protective barrier provided by the slimy coating due to stress of zinc salt results in subsequent wear and tear of the superficial cell layer of the outer (not inner) opercular covering. Regeneration of new MCs re-establishes the much desired protective mucoid covering, which helps the outermost layer of the epidermis to regenerate and smoothen. Isolation and bulging out of the ECs from their neighbours with their subsequent sloughing, singly or in batches of 2 to 3 cells are the main toxic manifestation of the zinc-salt on the outer opercular lining. These cells bear well dilated large, lightly stained nuclei. The inner epidermal lining of the operculum does not show similar massive wear and tear of the cellular constituents because this layer has more effective mucogenic activity than that of the outer covering. Even after 45 days of exposure when the outer opercular covering due to loss of mucous

coating shows extensive damage, including splitting, the density of MCs of inner opercular lining remains greater than that of the control level, causing no visible alteration to its structure.

Appearance of prominent intercellular vacuoles is an important symptom of disintegration of CCs. Following exposure to sublethal concentration of zinc chloride, the CCs also exhibit extensive damage in the form of marked vacuolization which is more severe than that of the acute treatment. Periodic regeneration of the CCs, however, also takes place and at certain stages of exposure (e.g. 30 days) more than one tier of CCs are established at certain sites. Prolonged exposure to the sublethal concentration of zinc chloride causes decreased density/size of the CCs which disappear following their damage. These damaged CCs later get invaded by phagocytes (Figure 1 *f*) for accelerating the process of degeneration of the CCs. Similar invasion of phagocytes within the CCs has recently been observed in the ammonium sulphate-treated fish[28]. However, infiltration of the CCs by phagocytes following exposure to mercuric chloride[1], sodium chloride[29] and detergents[30,31] has not been reported. While studying the response of CCs in the skin of the carp *Cyprinus carpio* to exogenous stresses (including heavy metals also), Iger *et al.*[32] noticed involvement of CCs in the lysis of leukocytes, probably after phagocytosis of these cells. They also noticed increased activity of the CCs of the exposed fish. Similar periodic alterations in the density of MCs, CCs and melanophores have also been observed under the toxic stress of several ambient xenobiotics (including heavy metal salts)[1,3,4,7,30,31,33–35]. Acute exposures to mercuric chloride and ammonium sulphate[24] induced great shedding of so-called still living ECs following their extensive damage at the outermost layer. This may cause indirect rupture of the CCs, leading to squeezing out of the contents of the underlying CCs due to lateral pressure of the neighbouring cells that actively contributes to lay a thick protective crust of proteinaceous substance over the still intact (apparently undamaged) epidermis to prevent further penetration of the ambient xenobiotics. Similar squeezing of CC materials was, however, not observed in the mechanically injured epidermis during wound healing experiments[36–38]. On the other hand, the CCs of dorsal skin of *H. fossilis* exposed to sublethal concentration of ammonium sulphate showed progressive decrease in the size due to waning of these cells during their migration to the surface becoming very small before being shed individually or along with other cellular elements[28]. According to Iger *et al.*[32] most phagosome-containing CCs finally migrate, actively or through the pressure of neighbouring cells, to the surface and ap-

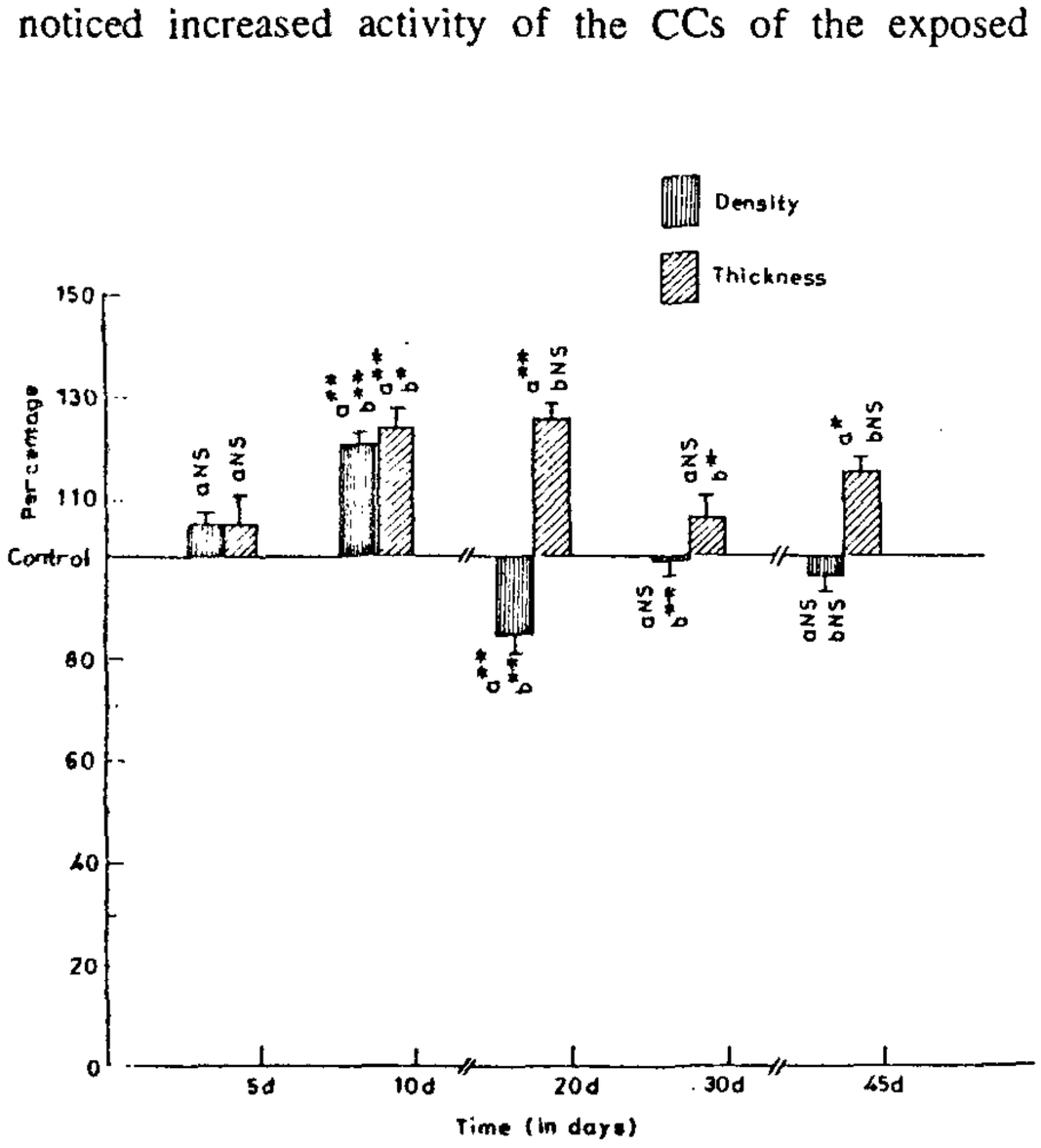

Figure 2. Periodic fluctuations in the percentages of density of polygonal epithelial cells (ECs) and thickness of the outer opercular epidermis at different stages of sublethal zinc chloride exposure. X SEM; based on Duncan's multiple range test. (Average of control values is considered as 100%. a, control VS respective experimental group; b, respective experimental group VS preceding experimental group; NS, non significant; *, $P < 0.005$, **, $P < 0.001$).

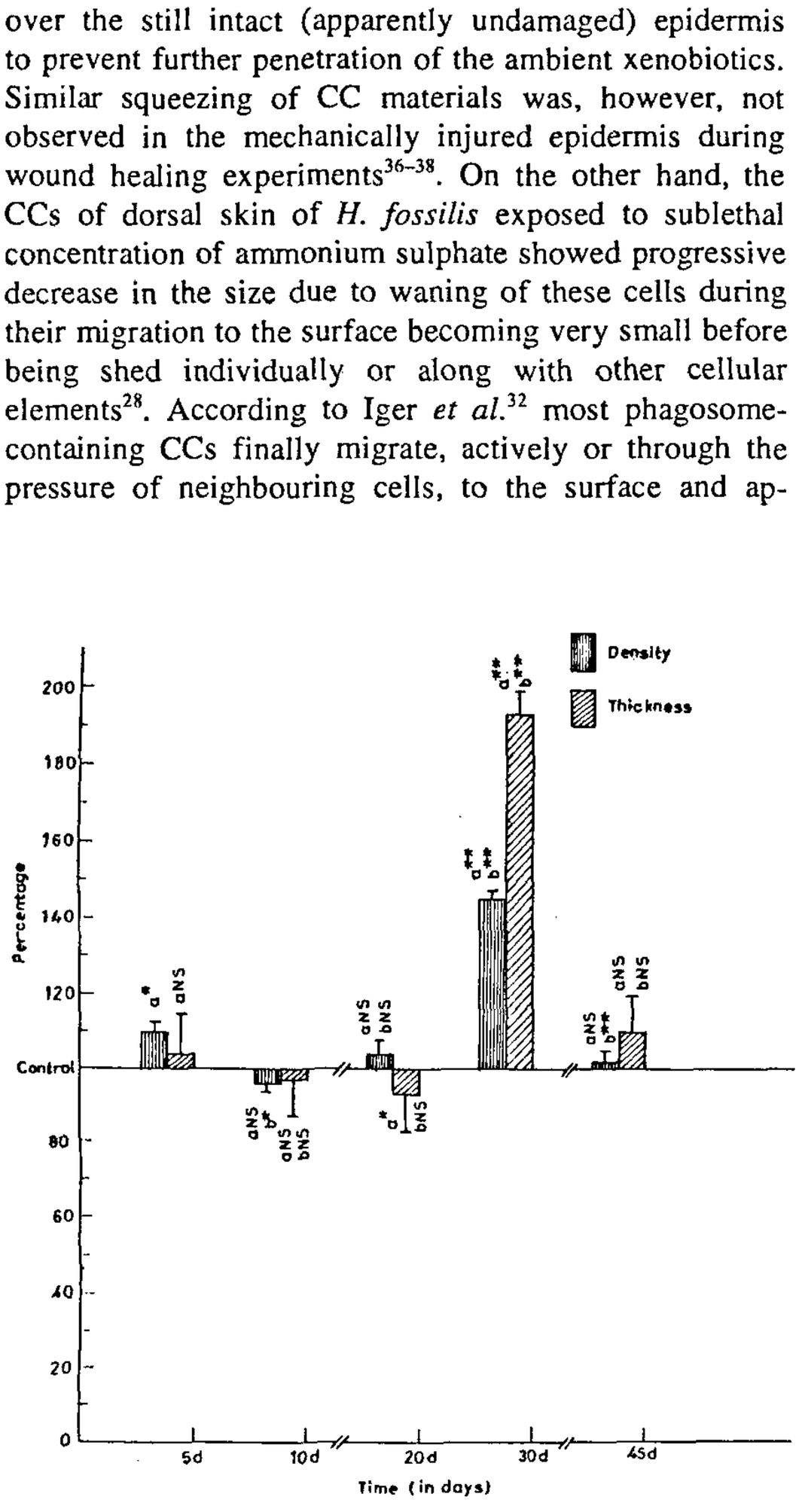

Figure 3. Periodic fluctuations in the percentages of density of polygonal epithelial cells (ECs) and thickness of the inner opercular epidermis at different stages of sublethal zinc chloride exposure. X SEM; based on Duncan's multiple range test. (Average of control value is considered as 100%. a, control VS respective experimental; b, experimental group VS preceding experimental group; NS, non significant; *, $P < 0.005$, **, $P < 0.001$).

parently leave the epidermis. They concluded that CCs are engaged in the stress response of fishes in addition to production of pheromones reported in the literature. Appearance of glycogen granules in the CCs of fish skin has also been observed following hyper-osmotic stress of ammonium sulphate[28], sodium chloride[29], and synthetic detergent[31]. ECs of detergent-exposed outer epidermal lining[31] and ammonium sulphate-treated inner epidermal (opercular) lining and regenerating dorsal skin epidermis following mechanical wounding also contain varying amounts of glycogen granules[24,39]. These authors correlated such accumulation of glycogen with disturbance of the normal physiology of the epidermis induced by the ambient toxicants and/or mechanical injury. However, exposure to mercuric chloride[1] and sublethal zinc chloride (present study) did not exhibit such deposition of glycogen granules in any of the cellular components of the outer opercular epidermis. Appearance of glycogen granules in the ECs of inner opercular epidermis after exposure to lethal concentration (only), however, indicates greatly disturbed physiology of these cells under the severe toxicity of the concentrated zinc salt solution. From the above-mentioned study, it is clear that sublethal concentration of zinc chloride solution causes certain prominent histopathological damages to the opercular epidermal linings which, in turn, can be considered for their utilization as potential bioindicator for analysing the variously contaminated waters.

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