Ammonium sulphate induced stress related alterations in the opercular epidermis of the live fish

*Heteropneustes (Saccobranchus) fossilis* (Bloch)

V. I. Paul and T. K. Banerjee
Centre of Advanced Study in Zoology, Banaras Hindu University, Varanasi 221 005, India

Histopathological analysis of the sublethal toxicity induced by 0.2 g/l (10% of 96 h LC₅₀ value) of the inorganic fertilizer ammonium sulphate to the outer and inner opercular epidermis of *Heteropneustes fossilis* has been made. Density and dimension of the goblet mucous cells (MCs) of the outer opercular epidermis increase enormously in the initial stages of exposure. Perinuclear vacuoles appear in the necrotic epithelial cells (ECs) which also bear pyknotic nuclei before their shedding at several stages of treatment. The club cells also exhibit great vacuolization. The damage becomes more extensive in later stages of exposure when severe wear and tear of the epidermis take place. The inner opercular lining however does not show such massive necrotic changes. Hyperplasia of the ECs and great vacuolization at various stages of exposure are the main histopathological alterations.

Histopathological studies pertaining to the toxicity of ammonia (NH₃ and NH₄⁺) are mainly concerned with the gills and certain other important organs like kidney, gonads, liver, alimentary canal1-5. While majority of the above organs are situated in the body cavity, the gills remain enclosed in the opercular cavity, which however allows the gills the direct exposure to the external environment. The operculum remains guarded by a protective lining of epidermis on its external and internal surfaces. Although the main function of the operculum is to facilitate irrigation of the gills, the epidermal linings play a vital role in maintaining the *milieu intérieur* of the fish by testing the quality of water. To understand the role played by the opercular epidermis of *H. fossilis* in combating the toxicity of the inorganic fertilizer, ammonium sulphate, this work was undertaken. Ammonium sulphate readily dissolves in water to form ionized (NH₄⁺) as well as unionized (NH₃) ammonia6.

Healthy individuals of *H. fossilis* (length 16-18 cm; body weight 35-40 g) collected from a single population at Varanasi were acclimated in large plastic aquaria for 3 weeks. Fish were fed with minced goat liver on every alternate day. Water was renewed after every 24 h, leaving no faecal matter and unconsumed food. For histopathological analysis, five groups of ten fish each were exposed separately to 501 of 0.2 g/l [10% of 96 h LC₅₀ value (2 g/l) determined by trimmed Spearman-Karber (with 5% trimming) method] and 24 h renewal bioassay system ammonium sulphate solution prepared in tap water having pH 7.5 dissolved oxygen 6 mg/l, waterhardness 23.2 mg/l and water temperature 22 ± 1°C. In the appropriate control groups, no ammonium sulphate was added. Experimental and control media were renewed after every 24 h. Feeding was allowed for control and experimental groups for 3 h before the renewal of the media. Five experimental and five control fish each were sacrificed after 5, 10, 20, 30 and 45 days of exposure. Opercula were fixed in 10% neutral formalin, Bouin’s fluid and Helly’s fluid. Six µm paraffin sections were stained with Ehrlich’s haematoxylin/eosin (H/E) for routine histopathological analysis, periodic acid Schiff (PAS) ⁸ for neutral glycoproteins, alcian blue pH 1.0 (AB 1.0) ⁹ for sulphated mucopolysaccharides, alcian blue pH 2.5 (AB 2.5) for acidic glycoproteins, AB 2.5/PAS dual staining for neutral and acidic glycoproteins¹¹ and bismarck brown (BB) for water-resistant mucopolysaccharides¹². Morphometric measurements were taken using an oculometer and stage micrometer. Standard statistical procedures based on random sampling from 10 different sections from all the five fish of each stage of all the experimental and control groups were performed. One way analysis of variance (ANOVA) followed by Duncan’s multiple range test were done for multiple comparison (Figures 1 and 2). Since the differences between the measurements taken from various control groups at different time intervals of the exposure were not significant, average of all the control groups was taken into consideration.
The outer opercular epidermis (EPD) is a stratified epithelium consisting mainly of sac-like goblet mucous cells (MCs) in the outermost layer (OL) and a single layer of large sized club cells (CCs) in the middle layer (ML). The basal layer is made up of epithelial cells (ECs). ECs also occupy the rest of the space (between the gland cells) of the epidermis. On the other hand, the inner opercular EPD does not possess any CC and is comparatively very thin. Rest of the cell types and their distribution are more or less identical to those of the outer opercular epidermis. The various histopathological alterations observed in the outer and inner opercular linings at different stages of exposure have been summarized in Tables 1 and 2. However with AB 1.0 (for sulphated mucosubstances) and saliva/PAS (for glycogen) methods the control and experimental epidermal linings of both the opercular surfaces gave negative reaction.

On transferring the fish into the test solution, MCs of both the opercular linings show hyperplasia and secrete copious amounts of slime. The additional quantity of mucus over the epidermal surface provides a thick barrier coating. After emptying, these cells degenerate and get lost, causing periodic decrease in their density, only to redevelop subsequently from the ECs of the lower layers as the next crop of MCs since the rate of disappearance of MCs is much faster than their regeneration. This is perhaps the reason for the periodic increase followed by decreased density of the MCs at different stages of exposure. The MCs, which usually differentiate in the lower layers of the epidermis, migrate to the surface to replace the degenerating goblet cells following their hyperactivity, exhaustion and emptying. Similar periodic fluctuations in the density of the MCs following exposure to ammonia solutions have also been observed in the gills and accessory respiratory organ. While studying the toxicity of organically fertilized water Iger et al. noticed complete depletion of MCs from the epidermis of exposed Cyprinus carpio between 3rd and 4th days of treatment. Exhaustion of mucus supply was also observed from the skin of Onchorhynchus mykiss exposed to acidified water. These authors however did not apply any statistical and/or biochemical
Figures 3-10. 3, Vertical section (VS) of the outer opercular epidermis of control *H. fossilis* showing normal histological organization; (H/E, \times 475). (BL = basal layer; CC = club cell; EC = polygonal epithelial cell; MC = mucous cell; ML = middle layer; OL = outer layer). 4-7. VS showing toxicopathological effects of sublethal concentration of ammonium sulphate in the outer opercular epidermis; (H/E, \times 475). 4, Increased density (number) and height of MCs of the OL after 5 days of exposure. Note the decreased size of CCs; 5, Sloughing of ECs in small flakes from the surface of the epidermis after 20 days of exposure. Note the active evacuation of the slime by a MC (arrow); 6, Appearance of prominent vacuole around the nucleus of the ECs at the OL. Prominent central vacuoles are seen in the CCs also after 30 days of exposure; 7, Massive wear and tear of the OL of the epidermis causing severe damage after 45 days of exposure. Note the prominent non-tissue spaces in the OL. 8, VS of a part of the inner opercular epidermis of control fish (EC = polygonal epithelial cell; MC = mucous cell); (H/E, \times 475). 9, 10. VS showing toxicopathological effects of sublethal concentration of ammonium sulphate in the inner opercular epidermis; (\times 475); 9, Hyperplasia of bismarck brown positive MCs indicating the presence of water stable mucoproteins after 5 days of exposure. This is in contrast to the negative reaction exhibited by the MCs of the control inner epidermis and also by both control and experimental outer epidermis; (BIB); 10, Appearance of prominent extra-cellular vacuoles mostly in lower layers of the epidermis after 20 days of exposure; (H/E).
Table 1. Summary of histopathological alterations in the outer opercular epidermis induced by sublethal concentration of ammonium sulphate

| Control EPD  
<table>
<thead>
<tr>
<th>Figure 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCs: sac-like mostly located in OL; weak to moderate AB 2.5, moderate to strong PAS, negative BB (also in exposed groups) reactions. CCr: characteristic perinuclear vacuoles with more than one nuclei. ECs: at MSL strong AB 2.5, faint PAS, weak BB reactions; at lower layers all reactions almost negative. Shape usually polygonal in OL, vertically compressed in ML, columnar in BL.</td>
</tr>
</tbody>
</table>
| Exposed EPD  
| 5 days  
| Figures 1, 4 | 
| MCs: density, size/height increase causing increased OL thicknesses; AB 2.5 reaction decreases; PAS reaction increases; new MCs regenerate in ML. CCr: size decreases; perinuclear vacuoles disappear. ECs: AB 2.5, PAS reactions unchanged; BB reaction increases, OL appears spongy due to vacuolization; acquire round/polygonal shape in ML due to decreased CC occupied area. |
| 10 days  
| Figure 1 | 
| MCs: density, size/height decrease causing decreased OL thickness; AB 2.5 reaction increases; PAS reaction decreases. CCr: size increases; perinuclear vacuoles reappear. ECs: AB 2.5 reaction increases; PAS reaction unchanged; BB reaction decreases to control level. |
| 20 days  
| Figure 5 | 
| MCs: density decreases; size/height decrease; AB 2.5, PAS reaction decreases. CCr: size increases further with decreased perinuclear vacuoles. ECs: AB 2.5 reaction decreases; PAS, BB reactions unchanged; slough regularly at MSL singly or in flakes. |
| 30 days  
| Figure 6 | 
| MCs: density increases greatly; size/height increases; AB 2.5 reaction unchanged; PAS reaction increases. CCr: perinuclear vacuoles reappear. ECs: AB 2.5 reaction decreases; PAS reaction increases; BB reaction unchanged. OL greatly vacuolated; cells show necrosis; sloughing of 'still living cells' at OL. |
| 45 days  
| Figures 1, 7 | 
| MCs: density decreases; size/height increase; AB 2.5 reaction increases; PAS reaction decreases. CCr: perinuclear vacuoles almost disappear. ECs: AB 2.5 reaction decreases; PAS reaction increases; BB reaction unchanged. OL middle layer, MSL = middle layer; MSL = most superficial layer; OL = outermost layer; PAS = periodic acid-Schiff. |

Abbreviations: AB 2.5 = alcian blue pH 2.5; BB = bismark brown; EC(s) = epithelial cell(s); CC(s) = club cell(s); EPD = epidermis; MC(s) = mucous cell(s); ML = middle layer; MSL = most superficial layer; OL = outermost layer; PAS = periodic acid-Schiff.

Table 2. Summary of the histopathological alterations in the inner opercular epidermis induced by sublethal concentration of ammonium sulphate

| Control EPD  
<table>
<thead>
<tr>
<th>Figure 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCs: sac-like, located mostly in OL; moderate to strong AB 2.5, PAS and negative BB reactions. ECs: at MSL moderate AB 2.5, faint PAS and BB reactions; at inner layers all reactions almost negative.</td>
</tr>
</tbody>
</table>
| Exposed EPD  
| 5 days  
| Figures 2, 9 | 
| MCs: density, height increase causing increased EPD thickness; AB 2.5, PAS reactions increase; BB reaction develops (strongly). ECs: AB 2.5, PAS reactions decrease greatly; BB reaction unchanged. cell junctions loosen due to increased vacuolization; hyperplasia. |
| 10 days  
| Figure 10 | 
| MCs: density increases causing increased EPD thickness; AB 2.5, PAS, BB reactions decrease. ECs: AB 2.5 reaction decreases; PAS reaction increases; BB reaction unchanged; extensive vacuolization. |
| 20 days  
| Figure 10 | 
| MCs: density decreases; AB 2.5, PAS, BB reactions slightly increase. ECs: AB 2.5, PAS, BB reactions almost unchanged; vacuolization in OL decreases; hyperplasia. |
| 30 days  
| Figure 11 | 
| MCs: density decreases causing decreased EPD thickness; AB 2.5, BB reactions increase; PAS reaction unchanged. ECs: AB 2.5 reaction increases slightly; PAS, BB reactions almost unchanged; vacuolization diminished greatly. |
| 45 days  
| Figure 2 | 
| MCs: density decreases causing decreased EPD thickness; AB 2.5, BB reactions decrease; PAS reaction unchanged. ECs: AB 2.5, PAS, BB reactions decrease greatly; vacuolization increases. |

Abbreviations: AB 2.5 = alcian blue pH 2.5; BB = bismark brown; EC(s) = epithelial cell(s); EPD = epidermis; MC(s) = mucous cell(s); ML = middle layer; MSL = most superficial layer; OL = outermost layer; PAS = periodic acid-Schiff.

investigation on the density of MCs and/or quantity of slime production. Statistical and/or histopathological investigations with heavy metals\(^{16-18}\), fish disinfectant\(^{19}\) and ammonium sulphate (present study) although exhibited great fluctuations in the density and volume of these goblet cells at different stages of exposure, total depletion of MCs was never noticed. One of the most important roles of the enhanced mucogenesis of the epidermal linings of the operculum is perhaps the protection of the enclosed delicate gills (within opercular chamber) from the environmental hazards.

The ECs of the most superficial layer of outer and inner opercular linings also lay a thin layer of water stable mucoproteins (glycocalyx) which persists for longer period as a water-resistant barrier coating against environmental hazards. Staining of the MCs of the inner opercular lining for water stable mucoproteins following ammonium sulphate exposure indicates the mixing of additional quantity of these (water resistant) mucoproteins into the slime laid down in the opercular chamber of the exposed fish for continued retention of the slimy protective coating against the ambient ammonium sulphate solution because exposure to this irritant causes enhanced ventilation frequency along with ventilation volume for increased respiratory efficiency\(^{20-24}\) subjecting the opercular chamber to the enhanced risk of loss of slime due to desquamation. Addition of water stable mucoproteins to the slime perhaps delays its loss considerably by facilitating greater retention of mucus. The ECs at the outer surface begin to exfoliate singly or in
flakes during the earlier stages of exposure. The sloughing becomes more pronounced after 30 days when the secretory activity of these cells along with that of the MCs decreases significantly due to hyperactivity and exhaustion.

The inner opercular epidermis however does not exhibit similar wear and tear because the mucogenic capacity of this epidermal lining is much more prominent than that of the outer epidermal covering, for protecting the delicate gills lodged within the opercular chamber.

The outer opercular epidermis of the exposed fish shows significantly increased thickness at most of the stages of exposure. Such variations in the thickness of the cutaneous epidermis has also been observed following exposure to certain other ambient xenobiotics. The increase in the thickness is rendered by several factors which include increased number of ECs, increased height of MCs and/or CCs, appearance of non-tissue spaces between the cells of the epidermal cells and oedematous swelling of the epidermis. The increased thickness causes increased barrier distance between the ambient xenobiotics and the underlying intact cell layers.


ACKNOWLEDGEMENTS. This work was supported by UGC, New Delhi. VIP thanks CSIR, New Delhi for a fellowship.

Received 6 February 1996; revised accepted 26 April 1996