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Responses of skin mucous cells to aluminum exposure at low pH in Atlantic salmon (Salmo salar) smolts

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Abstract: Atlantic salmon (Salmo salar) smolts were exposed for 80 h to seven water qualities: pH 5.6 with 31 and 46 µg labile Al L⁻¹, pH 6.0 with 18 and 24 µg labile Al L⁻¹, and pH 6.2 with 12 and 18 µg labile Al L⁻¹ and control water at pH 6.8 and <10 µg labile Al L⁻¹. The three groups with the highest concentrations of labile Al (31 and 46 µg labile Al L⁻¹ at pH 5.6 and 24 µg labile Al L⁻¹ at pH 6.0) suffered high mortalities and showed a disturbance in osmoregulation and a massive secretion of mucus, as seen from a decrease in number of skin mucous cells. Furthermore, an increase in skin mucous cells containing acidic mucosubstances was observed. The loss of plasma chloride and skin mucous cells showed a significant linear correlation (R² = 0.68, p < 0.001). The increased secretion of mucus on skin and gills and the increase in acidity of mucosubstances are consistent with their prior presumed defensive role in binding of Al.

Introduction

Acidification of freshwater causes an increase in dissolved Al as a result of Al leaching from soil and sediment (Schofield and Trojnar 1980; Overrein et al. 1981). Although low water pH can have negative effects on fish physiology (Packer and Dunson 1970; Brown 1981) and low Ca concentrations exacerbate the toxicity of acidified freshwater (McDonald et al. 1980; Rosseland et al. 1989), Al is considered to be the main toxic component in acidified environments (Leivestad et al. 1987; McWilliams 1990; Spry and Weiner 1991). The toxicity of Al is dependent on its aqueous speciation which is dependent on pH, temperature, and the presence of organic and inorganic substances (Howells et al. 1990; Rosseland and Staurnes 1994). Inorganic monomeric Al is believed to be the most toxic Al species (Driscoll et al. 1980) which is the predominant monomeric Al fraction at pH 4.5–5.5. (Lydersen et al.1990).

However, recently it has been established that Al toxicity can also be high at high pH when Al-rich acid water mixes with neutral water. The rapid increase in pH in such a mixing zone causes Al polymerisation and subsequently severe stress and mortality in fish (Rosseland et al. 1992). For anadromous species such as the Atlantic salmon (Salmo salar), the smolt stage is considered particularly sensitive to low pH/Al exposure (Rosseland et al. 1992; Skogheim and Rosse1984; Rosseland et al. 19866; Skogheim and Rosseland 1986) because of the extensive changes in physiology for preadapting them to marine life (Staurnes et al. 1993).

Osmotic failure is considered to be one of the main toxic mechanisms in salmonids exposed to Al at low pH (Leivestad et al. 1987). A loss in plasma ions in low pH/Al stressed fish is, in the short term, mostly associated with an increase in blood haematocrit (Malte 1986; Audet and Wood 1988; Wood et al. 1988). Another prominent characteristic of fish exposed to low pH/Al is the stimulated secretion of mucus (Muniz and Iger et al. 1994) and gills (Mueller et al. 1991). The role of this mucus secretion and the change in chemical composition of mucus during acid exposure has been suggested to be an adaptive response to prevent osmoregulatory failure (Solanski and Benjamín 1982; Zuchelkowski et al. 1985; Jagoe and Haines 1990) or to reduce Al accumulation on the gill epithelium (Mueller et al. 1991; Wilkinson and Campbell 1993).

The mucification of acid-exposed fish in short-term exposures (within the first day of exposure) is correlated with a...
reduction in mucous cells due to the exhausted secretion of the mucous cells (Iger et al. 1988, 1994). In long-term exposures (over 4 d; Iger et al. 1994), the mucification is thought to be due to hyperplasia of mucous cells in skin and gills (Zuchelkowski et al. 1981; Jagoe and Haines 1990). To our knowledge, the early reduction in number of mucous cells has never been applied as a quantitative parameter of the mucus secretion in low pH/Al exposed salmonids during the early phase of exposure, i.e., when rapid ion loss takes place.

The objectives of this study were to (i) characterise the initial mucous secretion and composition of skin mucus after low pH/Al exposure, by quantification of skin mucous cells, and (ii) document a concentration–response effect on mucification in correlation with ion regulation in the early stages of different low pH/Al combinations.

Materials and methods

Experimental conditions and protocol

Fish used were 1-yr-old smolifying Atlantic salmon from the River Imsa strain. For smolts, mean ± SD of length (fork length), mass, and condition factor were 17.6 ± 0.7 cm, 49.8 ± 4.7 g, and 0.89 ± 0.04 g cm⁻², respectively (n = 210). These fish were raised at the Freshwater Research Station at Ims from the Norwegian Institute of Nature Research and were kept for 2.5 d before the experiment in their experimental tanks (80 fish per tank) in neutral water in order to acclimatize (pH 6.75, Ca = 3.56 ± 0.03 mg L⁻¹, total Al < 10 mg L⁻¹, temperature 10.5°C). In one of the experimental tanks, this water quality was maintained during the entire experiment as a control. The experimental tanks had a volume of 80 L with a flow rate of 2.3 L min⁻¹, creating enough countercflow to give the fish natural swimming movement. During the entire experiment (80 h) the fish were fed. A photoperiod regime of 16 h of light and 8 h of darkness was maintained.

Six different water qualities were made by mixing acid Al-rich tap water (pH 5.0, Ca = 1.8 ± 0.02 mg L⁻¹, total Al = 74.0 ± 0.02 mg L⁻¹, labile Al = 4.5 ± 0.03 mg L⁻¹) and tap water from the River Imsa (pH 6.8, Ca = 3.56 ± 0.03 mg L⁻¹, total Al < 10 mg L⁻¹, labile Al < 10 mg L⁻¹) in three separate main tanks (90 L), resulting in water with pH levels of 6.2, 6.0, and 5.6, respectively. From here, water from each main tank was distributed to two supplementary tanks (20 L), one of which was given extra Al (to give an addition of 23 µg total Al L⁻¹ in the exposure tanks). The water from these supplementary tanks was delivered to the exposure tanks. The succession of the main and supplementary tanks was distributed to two supplementary tanks (20 L), one of which was called nonlabile Al and is presumed to primary include organically complexed monomeric Al (Rosseland et al. 1986a). The concentration of labile or inorganic monomeric Al was calculated from the

Histology and tissue processing

A small piece of skin was taken from the ventral side near the head. This part of the skin has few scales and is therefore appropriate for histological analysis. Care was taken not to damage the skin during sampling. The skin samples were fixed for 24 h in Bouin’s fixative (volume ratio of picric acid – formaldehyde – acetic acid = 15:5:1); afterwards the samples were washed three times in 70% ethanol and preserved in 90% ethanol. After routine dehydration in alcohol series, the samples were embedded in paraffin wax (oriented vertically) and 5-mm cross sections were cut with a microtome (MÖD.1330/Biocut) (Ferguson et al. 1992). Special care was taken to cut the sections consistently in a plane perpendicular to the surface of the epidermis. The sections were stained with Alcian blue at pH 2.6 (AB.2), haemalum, and eosin. The AB.2 was used to identify the mucous cells containing acid glycoprotein (Jones and Reid 1973): AB.2-positive mucous cells have an acid, negatively charged mucus content, and AB.2-negative mucous cells contain more neutral mucosubstances. The sections were examined with a Zeiss light microscope at 400× magnification. A Mertz linear grid was used to, planimetrically, quantify the number of mucous cells per 0.1 mm transect of skin epithelium; 12 cross sections were examined for each fish, and six fish per group were examined randomly. Because the thickness of the epidermis in the exposed groups did not change (this was determined by measuring the epidermal height at both ends and in the middle of a 0.1-mm transect in 12 cross sections per fish), the density of the mucous cells was expressed as number of cells per 0.1 mm. Mucous cells were identified as large swollen cells with a basal located nucleus. Mucous cells with acid glycoproteins stained deep blue with AB.2.

Water chemistry

Temperature and pH were measured three times a day (Orion model 207 pH meter with a Radiometer glass electrode) during the experiment. For Al specification, water samples were collected (n = 3), stored at low temperature (4°C), and subjected to Al specification analyses in the laboratory of the Norwegian Institute for Freshwater Research according to the EN 45000 standards. Al was analysed by the pyrocatechol-violet method (Dougan and Wilson 1974) adapted for automatic analysis by Henriksen and Bergmann-Paulsen (1975), and the fraction obtained without any pretreatments was denoted acid-reactive Al. The acid-reactive fraction is presumed to contain the total monomeric Al. Water samples were fractionated over a cation-exchange column using the procedure of Driscoll (1980), modified for FIA by Rozeberg and Henriksen (1985). The Al in the eluate was called nonlabile Al and is presumed to primary include organically complexed monomeric Al (Rosseland et al. 1986a). The concentration of labile or inorganic monomeric Al was calculated from the

Table 1. Water chemistry (means ± SD, n = 3, temperature = 10.5°C) of the experimental and reference water.
Fig. 1. Plasma Cl\(^{-}\) (A) without and (B) with an addition of 23 mg total Al L\(^{-1}\) and haematocrit (C) without and (D) with an addition of 23 mg total Al L\(^{-1}\) (mean ± SD, n = 6) for smolts of Atlantic salmon exposed for 80 h to low pH/Al water. Lines with open symbols represent the groups with significantly different values from the values of the control group: *p < 0.05; **p < 0.01.

Statistics

Data are presented as means ± SD. Overall differences between groups were assessed by analysis of variance (ANOVA), with time and treatment (pH, Ca, and Al addition) as independent variables. Significance was tested using Tukey's test (p < 0.01 and p < 0.05). Best correlation between variance and factors (pH, Ca, acid-reactive Al, nonlabile Al, and labile Al) was assessed by matrix correlation followed by multiple regression analysis. The correlation curves were fitted with the regression program FIG.P. 6.0.

Results

No mortality was observed for fish held in pH 6.8 (control), pH 6.0, and pH 6.2/low Al (<10, 17.5, and 11.5 μg labile Al L\(^{-1}\), respectively). The addition of Al at pH 6.0 (23.5 μg labile Al L\(^{-1}\)) resulted in a LT\(_{50}\) of 36 h. Both the pH 5.6/low and high Al groups (31 and 45.5 μg labile Al L\(^{-1}\), respectively) showed high mortality, with LT\(_{50}\)'s of 60 and 29 h, respectively.

At the start of the experiment the plasma Cl\(^{-}\) and haematocrit levels were 130 ± 5 mmol L\(^{-1}\) and 43 ± 7%, respectively. Plasma Cl\(^{-}\) and haematocrit levels in control fish did not change significantly during the experiment. After 16 h the pH 5.6/low and high Al and the pH 6.0/high Al groups had a significantly lower plasma Cl\(^{-}\) than controls and remained low during the rest of the experiment (Figs. 1A and 1B). In all exposure groups the haematocrit values increased initially, reaching highest values after 15 h after which they returned to control levels (Figs. 1C and 1D). The pH 6.0/high Al and pH 5.6/low and high Al groups differed significantly from the controls after 7 h (Fig. 1C).

The mucous cells in the skin of our Atlantic salmon corresponded with the description Daye and Garside (1980) for this species. The total number of skin mucous cells showed a rapid initial decrease in the first 16 h for all groups, except the controls and the pH 6.2 and pH 6.0/low Al groups. The rapid decrease slowed down later (Figs. 2A and 2B). After 16 h of exposure in the pH 5.6/low and high Al and the pH 6.0/high Al groups, the number of skin mucous cells was significantly lower than in controls; the reduction in skin mucous cells was 40% for both the pH 5.6 and pH 6.0/high Al groups and 27% for the pH 5.6/low Al group. Along with the loss of skin mucous cells, the Atlantic salmon smolts held in the three acute toxic water qualities (pH 5.6/high and low Al and pH 6.0/high Al) developed a visible thick layer of mucus on the skin and coagulates of mucus on the edges of the opercula within the
Fig. 2. Number of mucous cells in the skin (A) without and (B) with addition of 23 mg total Al·L\(^{-1}\) and ratio of AB.2-positive to AB.2-negative mucous cells (C) without and (D) with addition of 23 mg total Al·L\(^{-1}\) (mean ± SD, n = 6) for smolts of Atlantic salmon exposed for 80 h to water of different pH and Al concentrations. Lines with open symbols represent the groups with significantly different values from the values of the control group: *p < 0.05; **p < 0.01.

Fig. 3. Correlation between plasma Cl\(^{-}\) concentration and number of skin mucous cells; regression line: skin mucous = 0.61 Cl\(^{-}\) - 3.24, \(R^2 = 0.68, p < 0.001\).

Both the decrease in number of mucous cells and the loss of plasma Cl\(^{-}\) were correlated with an increase in labile Al (multiple regression analysis, \(p < 0.01\)). Plasma Cl\(^{-}\) levels decreased in direct proportion to the decrease in the number of mucous cells (Fig. 3A; \(R^2 = 0.68, p < 0.001\)).

Discussion

At the start of the experiment the plasma Cl\(^{-}\) and haematocrit levels were similar to those in smolts of the same strain in neutral water (Rosseland and Skogheim 1984). The loss of plasma Cl\(^{-}\} and increase in haematocrit were already initiated at labile Al concentrations of 24 mg·L\(^{-1}\) at pH 6.0 and a Ca concentration of 2.35 mg·L\(^{-1}\). These results agree with the study of Rosseland et al. (1986a), who documented plasma Cl\(^{-}\) loss (from 110 to 85 mmol·L\(^{-1}\)) for Atlantic salmon smolts exposed to 21.5 mg labile Al·L\(^{-1}\) at pH 6.6 and 2.68 mg Ca·L\(^{-1}\). The mixing of acid-rich tap water with neutral river water in the supplementary tanks implies that we cannot exclude a possible toxic influence from ongoing Al polymerisation (Rosseland et al. 1992). However, this is unlikely because no toxic effects were observed in brown trout (Salmo trutta) exposed to a 5.6-min aged mixing zone (Verbost et al. 1995). The retention time of 20 min of the mixed water in this experiment was considered long enough to diminish possible toxic effects of mixing before the water entered the exposure tanks.
In addition, both the decrease in number of mucous cells and the loss of plasma Cl⁻ were significantly \((p < 0.01)\) correlated with an increase in labile Al. The labile Al concentration in the exposure tanks was therefore considered to be the predominant toxic Al fraction.

High mortalities were observed in the three exposure groups with the highest concentrations of labile Al \((\text{pH 5.6/low and high Al and pH 6.0/high Al})\). These groups were also characterised by a disturbance in osmoregulation, (decrease in plasma Cl⁻ values with a subsequently increased haematocrit) and a reduced number of skin mucous cells. No significant difference in plasma Cl⁻, haematocrit, or number of skin mucous cells was observed between these three groups, suggesting that these concentrations of labile Al caused a maximum stress response resulting in high mortality.

The reduction in number of mucous cells is probably the result of exhaustion of the mucous cells caused by extensive mucus secretion (Iger et al. 1988, 1994; Roy 1988). The strong decrease in skin mucous cells was associated with a general mucification of gills and skin, and this indicates that the number of skin mucous cells is a good parameter for quantification of mucus secretion.

The initial rapid exhaustion of the mucous cells is apparently not compensated by formation of new skin mucous cells within the 80 h of exposure. This indicates a long generation time, and this is in line with data from the literature. Generation of new skin mucous cells takes 5–6 d in carp \((Cyprinus carpio)\) (Iger et al. 1988), Arctic char \((Salvelinus alpinus)\) (Pickering and Macey 1977), and brown trout (Pickering et al. 1982). Other authors, however, observed hyperplasia within the first 2 d of acid exposure in the gills of rainbow trout \((Oncorhynchus mykiss)\) (Audet and Wood 1993) and in the skin of brown bullhead \((Ictalurus nebulosus)\) (Zuchelkowski et al. 1986). The observed increase in mucous cell numerical densities in these studies might reflect a proliferation of immature mucous cells which is not dominated by a loss of mucous cells due to a massive discharge of their contents. Iger et al. (1994) described a first-phase response of skin mucous cells in acid-exposed fish by migration of the mucous cells towards the epidermal surface combined with increase in cell volume. This could result in an increase in the number of identifiable mucous cells. If the rapid depletion of the mucous cells is not compensated for by formation of new cells, this will lead to reduced mucus secretion. Since body mucus has an important role for protection against pathogens or toxicants (Shephard 1994), exposure to low pH with Al could make the fish more vulnerable to these agents.

The groups with a significantly fewer number of mucous cells also had an increased ratio of AB.2-positive to AB.2-negative cells, indicating a relative increase in skin mucous cells containing acid glycoproteins (Ferguson et al. 1992). In contrast, Iger et al. (1994) reported an increase of mucous granules with more neutral glycoproteins in the skin of rainbow trout exposed to acid water. However, in their study the increase in mucous granules with neutral glycoproteins occurred after 4 d of exposure whereas the relative decrease in neutral glycoproteins in our study was observed within the first 3 d of exposure. Because mucus is initially synthesised as neutral glycoproteins (Mittal et al. 1980) and the differentiation of new mucous cells takes 5–6 d (Pickering and Macey 1977; Pickering et al. 1982; Iger et al. 1988), the increase in neutral glycoproteins in the studies of Iger et al. (1994) might reflect a differentiation of new mucous cells after depletion of mucous cells in the first phase of exposure. Reid et al. (1991) found a reduced gill sialic acid content after 8 d of low pH/Al exposure in rainbow trout. Because sialoglycoproteins are a major component of the mucus layer (Fletcher et al. 1976), this reduced gill sialic acid might reflect a relative increase in neutral glycoproteins similar to the observations of Iger et al. (1994). The accuracy of measurement of mucus content, by a component such as sialic acid (Harris et al. 1973), may be influenced by changes in the chemical composition of the mucus (McDonald et al. 1991). The change in ratio in our study could reflect stimulated differentiation of new mucous cells with acid-rich glycoproteins or an increase of acid glycoproteins in already formed mucous cells. The latter process was the most likely in the present study because the differentiation of new mucous cells took longer than the 80 h of exposure time. Mittal et al. (1980) stated that fully differentiated mucous cells can increase the degree of sulfating of their glycoproteins. In addition, Zuchelkowski et al. (1985) found an increased sulfation of mucosubstances after 24 h in acid-exposed brown bullhead. This process of enhanced sulfation in mature mucous cells will subsequently lead to an increased release of mucus containing more acid glycoproteins.

In earlier studies on brook trout \((Salvelinus fontinalis)\) exposed to low pH/Al, Booth et al. (1988) divided the ionoregulatory response into two relatively distinct phases: an initial "shock" phase characterised by a loss in blood ions and a "recovery" phase where surviving fish showed some recovery. In our study the shock phase was characterised by a rapid loss in plasma Cl⁻ and a subsequent increase in haematocrit during the first 16 h, with no recovery of the plasma Cl⁻ values during the remainder of the exposure period. The ion-regulatory shock phase of low pH/Al exposure appeared to be associated with the decrease in the number of mucous cells. A linear correlation \((R^2 = 0.68, p < 0.001)\) was found between the decrease in plasma Cl⁻ (indicating osmoregulatory failure) and the decrease in the number of skin mucous cells.

These results coincide with the presumed adaptive function for mucus secretion in the response of fish to low pH/Al exposure, as was suggested before by several authors (Shephard 1994). A function of mucus in prevention of an osmotic disturbance by Al exposure, e.g., by forming a diffusion barrier to passive plasma ion movement across the skin and gill membrane or to concentrated cations near the epithelial surface to aid the active uptake of ions, seems to be unlikely because Na⁺,Cl⁻, and Ca²⁺ diffusion is not substantially affected by mucus layers, as measured on dilute solutions of mucus (Marshall 1978; Pärt and Lock 1983; Stith 1984). More likely, mucus has an indirect function by retarding Al diffusion from the solution to the membrane surface, thus reducing the negative impact of Al on the epidermal surface (Wilkinson and Campbell 1993). Wilkinson and Campbell (1993) observed a high absorption of gill-associated Al in the mucus and not to the gill surface itself, resulting in the largest accumulation of Al in the mucus layer. This accumulated Al can be readily eliminated from gill surface by high secretion of mucus (McDonald et al. 1991; Mueller et al. 1991) coupled with increased rate of mucus sloughing (Levestad 1982; Neville and Campbell 1988; Wilkinson and Campbell 1993), thus reducing Al damage or accumulation on the gill surface that may cause the ionoregulatory
disturbance. The relative increase in AB₂-positive mucous cells observed in our study could subsequently lead to secretion of more negatively charged mucus. Since most accumulated Al is probably absorbed to the gill surface by binding to negatively charged groups of the mucus layer (Reid et al. 1991; Wilkinson and Campbell 1993), the relative increase in mucous cells containing acid glycoproteins could enhance the retention of Al in the mucus layer.

Excessive secretion of mucus, together with a precipitation of Al, however, might lead to clogging of the gills and respiratory problems (Leivestad 1982; Witters et al. 1996). The greater mucus production with deposition of Al can increase the resistance to O₂ and CO₂ diffusion and subsequently lead to respiratory dysfunction (Witters et al. 1996). Leivestad (1982) found an excessive mucus secretion and clogging on the gill with hyperventilation and reduced venous oxygen pressure in low pH/Al exposed brown trout. In addition, Witters et al. (1996) observed an increased ventilation frequency with gill mucification after exposing brown trout to Al in a toxic mixing zone.

The present study showed that the decrease in the number of skin mucous cells is associated with the overall mucification of skin and gills in the first phase of Al exposure, which is characterised by a rapid disturbance of ion regulation. The increase in secretion of mucus and change in mucus chemistry support the presumed defence role of mucus against Al toxicity.

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