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Low levels of vitamin E in plasma from Atlantic salmon *Salmo salar* with acute infectious pancreatic necrosis (IPN)

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ABSTRACT: Blood and pancreatic tissue from Atlantic salmon *Salmo salar* L. postsmolts were sampled from 5 sea water fish farms in Norway during clinical outbreaks of IPN. Formalin-fixed pancreatic tissue was examined histologically. The presence of IPN-virus (IPNV) in pancreatic lesions was demonstrated at all farms by means of immunohistochemistry. IPNV was isolated in cell cultures, both from kidney and pancreatic tissue sampled from 2 of the farms. α-Tocopherol in blood plasma was measured by HPLC. The levels of α-tocopherol in blood plasma from Atlantic salmon with acute IPN were significantly lower than in healthy fish sampled from the same cages. We conclude that vitamin E levels cannot be used to differentiate IPN from pancreas disease.

KEY WORDS: Infectious pancreatic necrosis · Atlantic salmon · Vitamin E · α-Tocopherol · Pancreas disease

INTRODUCTION

IPNV and IPN in Norway

Infectious pancreatic necrosis virus (IPNV) was isolated for the first time in Norway in 1975, from rainbow trout *Oncorhynchus mykiss* (Håstein & Krogsrud 1976). There has been a rapid increase in the incidence of IPNV isolations since 1982 (Krogsrud et al. 1989). The virus is now considered endemic in salmon sea water farms in Norway (Melby et al. 1991).

The first clinical outbreak of IPN in Norway was diagnosed in 1985 in Atlantic salmon *Salmo salar* fingerlings (Krogsrud et al. 1989). In the following years, the disease was recorded in a few hatcheries only, all with limited losses. Nevertheless, an eradication program was carried out at the 2 major breeding stations. Broodfish and fry were tested for the presence of IPNV and positive groups were culled.

The incidence of the disease increased rapidly during the years 1989 to 1992 in spite of the measures mentioned above, and IPN is now considered a significant economic problem in the fish farming industry. A remarkable shift in the disease pattern has also been observed. While IPN formerly was a disease of fry and fingerlings, it is now commonly diagnosed in postsmolts during their first months in sea water.

Pancreas disease

Pancreas disease (PD) is a condition affecting farmed Atlantic salmon, and the disease is a particular problem in Scottish and Irish sea-caged sites (Munro et al. 1984, McVicar 1987, Murphy et al. 1992). Affected fish have lowered plasma and liver vitamin E levels (Bell et al. 1987), both down to only 7% of the levels in healthy Atlantic salmon of the same age and under the same rearing conditions (Ferguson et al. 1986), leading to

the suggestion that vitamin E is involved in the pathogenesis of PD. The results of epidemiological and transmission studies do however suggest that PD has an infectious etiology (McVicar 1987, Raynard & Houghton 1993, Houghton 1994), although diets with high levels of polyunsaturated fatty acids together with vitamin E may have a protective action (Raynard et al. 1991).

IPN and PD

One of the most prominent pathological changes in acute phases of both IPN and PD is extensive necrosis of exocrine pancreatic tissue (McKnight & Roberts 1976, Munro et al. 1984). However, the patterns of change associated with the 2 diseases are different (McVicar 1987). Both diseases can develop into chronic stages with pancreatic fibroplasia (McKnight & Roberts 1976, Munro et al. 1984, McVicar 1987). The similarities in pathomorphology between the 2 diseases have given rise to confusion, especially when dealing with chronic cases. As most sea water farms in Norway harbour clinically healthy carriers of IPNV (Melby et al. 1991), the mere isolation of IPNV during a disease outbreak is of limited diagnostic value.

We decided to measure the levels of vitamin E in plasma from Atlantic salmon postsmolts during outbreaks of IPN. This was done to determine whether low vitamin E levels are associated with this viral disease that also targets the pancreas, and also whether vitamin E levels can be used as a tool to differentiate IPN and PD.

MATERIALS AND METHODS

Disease outbreaks. In 3 farms (A, B & C), the fish had been transferred to sea water in spring, and disease outbreaks occurred in June. In the other fish farms (D & E), the fish were put to sea in autumn, and the disease outbreaks occurred from January to March. Samples of blood, pancreatic tissue and mid-kidney from 10 fish in farms A & B were collected, as well as samples of blood and pancreatic tissue from 46 additional individuals from farms A, B, C, D & E (a total of 66 salmon). From farms A & B, both healthy and clinically sick individuals from the same cage were sampled. From farms C, D & E, only clinically diseased fish were examined.

Pathology. At necropsy, pyloric ceca with attached pancreatic tissue were fixed in 10% phosphate-buffered formalin immediately after blood sampling. The samples were processed and embedded in paraffin wax according to standard routines. Sections (4 to 6 µm) were stained with haematoxylin and eosin (H&E) and examined by light microscopy. Pancreatic lesions were classified as shown in Table 1.

Immunohistochemical examination. Sections from all the paraffin wax-embedded tissues were examined to identify IPNV with slight modifications of the method described by Evensen & Rimstad (1990). After a blocking step with 5% bovine serum albumin (BSA) in Tris buffer, rabbit antiserum against IPNV serotype Sp was added to the tissue sections. The sections were rinsed and biotinylated goat anti-rabbit immunoglobulin was applied. After a second rinsing, avidin-biotin-complex (ABC) with alkaline phosphatase was added (Evensen 1993). Following another rinsing, the primary antigen/antibody reactions were made visible by adding Fast Red chromogen, followed by washing and counterstaining with Mayer's haematoxylin. The sections were examined by light microscopy.

Virus isolation and titration. Samples from mid-kidney and from pyloric ceca from 10 fish from farm A and 10 fish from farm B were homogenized separately in 1:10 dilution (v/v) in Glasgow modification of minimum essential medium (GMEM) without serum. The homogenates were clarified by low speed centrifugation. In order to determine the infectivity, 25 µl of 10-fold dilutions of the supernatants were inoculated in 3 parallel experiments onto CHSE-214 cells (Lannan et al. 1984), in 96-well microtiter plates maintained in GMEM with 2% inactivated bovine foetal serum at 15°C. IPNV from cell cultures showing cytopathic effect was identified with a fluorescent antibody technique (FAT) using a monoclonal antibody against IPNV protein 3 (Intervet Norbio, Bergen, Norway).

Blood sampling. Fish were stunned by a blow to the head. Blood samples were drawn from the caudal vein in heparinized evacuated blood collecting tubes. The blood was kept on ice until centrifugation, and plasma was then frozen at –70°C until examination could be carried out.

Vitamin E analyses. After precipitation of proteins with ethanol (+1% ascorbic acid), tocopherols in the plasma samples were extracted with hexane. The hexane was evaporated and the residue redissolved in ethanol. α-Tocopherol concentrations were measured by HPLC with reversed-phase C18 column and fluorescence detector. Calculations were based on internal standards added in all samples.

Statistics. The levels of α-tocopherol in plasma from fish with acute IPN were compared to the levels in plasma from healthy fish from the same fish farm applying the Wilcoxon Rank Sum test. The levels of α-tocopherol in plasma from fish with acute IPN from all farms were compared to the levels in plasma from all

healthy fish (farms A & B), using the Wilcoxon Rank Sum test.

RESULTS

Pathology

At necropsy, most of the clinically diseased fish had varying degrees of ascites, from negligible to pronounced. Most fish with ascites also had varying degrees of petechiation in the pyloric region, from barely visible to pronounced. The body muscle appeared dry. Four salmon had external wounds. Histological examination showed severe necrosis of exocrine pancreatic tissue in 30 of 66 samples (Table 1).

Immunohistochemistry

The presence of IPNV in pancreatic lesions appeared as small red cytoplasmic granules in degenerating and necrotic acinar cells. All sections with pancreatic changes consistent with the diagnosis of acute IPN stained positive (Table 1).

Virus isolation and titration

IPNV was isolated from all samples examined. Fish with acute IPN all had virus titers $\geq 10^{10.1}$ $TCID_{50}$ ml^{-1} both in sampled pancreas with pyloric ceca and in mid-kidney. Most healthy fish (8 out of 10) had virus titers below this.

Table 1. *Salmo salar.* Immunohistochemical identification of IPNV in pancreatic tissue in relation to pancreatic lesions

Pancreatic lesions	Immunohistochemical staining: IPNV-positive	IPNV-negative	Total no. of fish
Early lesions or mild lesions[a]	4	0	4
Acute IPN[b]	30	0	30
Subacute and chronic lesions[c]	2	6	8
No pancreatic lesions	0	24	24
Total no. of fish	36	30	66

[a]Moderate numbers of degenerating and pycnotic acinar cells affecting minor parts of pancreatic tissue
[b]Pronounced necrosis of exocrine pancreatic tissue showing condensed acinar cells, pycnosis and area(s) containing eosinophilic debris
[c]Mixed lesions with varying degrees of necrosis, inflammation and/or fibrosis

Table 2. *Salmo salar.* Plasma α-tocopherol (μg ml^{-1}) in Atlantic salmon postsmolts

	Farm A		Farm B		Farm C	Farm D	Farm E
	Acute IPN	Healthy	Acute IPN	Healthy	Acute IPN	Acute IPN	Acute IPN
	4.1	46.9	8.5	24.0	12.2	9.5	8.2
	12.1	40.1	7.2	33.3	1.8	16.1	5.3
	1.1	41.7	4.3	24.5	3.8	26.0	
	20.7	32.8	11.2	21.2	2.2	12.4	
	11.9	33.0		32.1	3.4[a]		
	19.2	42.7		48.7			
	18.7	47.8		23.3			
	17.8			31.5			
	10.3			25.9			
	17.7			20.6			
	12.0			16.9			
	13.3			24.5			
				15.8			
Avg	13.2	40.7	7.8	26.3	4.7	16.0	6.8
SD	5.59	5.20	2.48	8.27	3.83	6.23	1.45

[a]Pooled samples from 4 salmon

Vitamin E analysis

Levels of α-tocopherol in plasma from Atlantic salmon suffering from acute IPN were significantly lower than those from healthy fish from the same sea cages in both farm A ($p < 0.001$) and farm B ($p < 0.005$) (Table 2). Moreover, when comparing the level of α-tocopherol in plasma from all fish with acute IPN with the level in plasma from all healthy fish, these 2 groups were also found to be significantly different ($p < 0.001$). In general, fish with early and/or mild lesions had moderately low α-tocopherol levels (mean of 17 μg ml^{-1}), while fish with subacute or chronic lesions had markedly low levels (mean of 5.5 μg ml^{-1}).

DISCUSSION

In the present study, low levels of α-tocopherol were found in the plasma of Atlantic salmon with acute IPN. The drop in levels is statistically significant to a high degree of confidence, when comparing diseased and healthy fish from the same farm, as well as when comparing the healthy fish on farms A & B with the IPN-affected fish from all 5 farms.

In earlier studies (Poppe unpubl.), the mean level of α-tocopherol in plasma from 19 wild salmon was found to be 42 μg ml^{-1}, while the corresponding level in 98 healthy farmed Atlantic salmon was 22 μg ml^{-1}. In the present study, healthy fish from farm A had levels close to those seen in the wild fish, while the healthy fish

from farm B had levels comparable to the farmed fish. The amount of α-tocopherol in plasma and tissue is highly dependent on dietary levels, as well as on the amount and quality of dietary fat (Raynard et al. 1991, Bai & Gatlin 1993, Obach et al. 1993, Waagbø et al. 1993). The differences in α-tocopherol levels in the healthy fish in farms A & B may reflect dietary differences, although these were not measured.

In the present study, the diagnosis of IPN was based on histopathological criteria (McKnight & Roberts 1976), on immunohistochemical demonstration of IPNV in pancreatic lesions (Evensen & Rimstad 1990), and on demonstration of high virus titers in kidney and pancreas. These criteria clearly distinguish IPN from PD. However, as there is no published information on a specific test for the etiological PD-agent, and as PD has been reported in Norway earlier, we cannot rule out the possibility of the co-existence of the PD-agent in these disease outbreaks.

Research on the relation between infectious diseases and vitamin E levels has mainly been focused on the effects of dietary vitamin E on general immunity and the prevalence and severity of infectious diseases. Combined vitamin E/selenium deficiency will affect several parts of the immune system (Tengerdy 1980, Lessard et al. 1991), and vitamin E/selenium supplementation is reported to protect against or reduce the severity of several infectious bacterial diseases in mice (Tengerdy 1980), pigs (Teige et al. 1978), cattle (Hogan et al. 1993), and poultry (Tengerdy 1980, Latshaw 1991). The studies reported by Raynard et al. (1991) indicated that dietary vitamin E supplementation may also have a protective effect against PD in Atlantic salmon. Similar systematic studies on the effect of vitamin E supplementation on IPN have not been conducted so far.

The results of the present study indicate on the other hand that acute IPN may induce a lowering of vitamin E levels in Atlantic salmon. In mammals, including humans, disorders known to induce lowered vitamin E levels may be divided into 2 groups. The first group, thought to lower vitamin E levels through reduced absorption, includes chronic steatorrhoea because of pancreatic insufficiency in cystic fibrosis and some other malabsorption syndromes (Farrell 1980). The second group, where the deficiency is thought to result from increased α-tocopherol utilization in the body, includes congenital haemolytic anaemias like sickle cell anaemia and β-thalassemia, and the lipid storage disorder called Gaucher's disease (Rachmilewitz et al. 1982). The effect of IPN on α-tocopherol levels indicated by the present study may be a result of malabsorption due to impaired function of the diseased pancreas. However, the acute character of the pathological lesions and the apparently quick reduction in plasma α-tocopherol levels suggest that the effect is mainly the result of increased consumption of vitamin E. Lowered total vitamin E levels secondary to infection with influenza virus A have been reported in experimentally inoculated mice (Hennet et al. 1992).

In summary, the results of this study suggest that viral damage to the pancreas is associated with consumption of α-tocopherol. We do not know whether this is mainly an inflammatory-associated, virus-associated or a pancreas-associated phenomenon. We conclude that we cannot use vitamin E levels as a tool to help differentiate IPN from PD in postsmolts.

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