

Histopathology survey of rainbow trout (*Oncorhynchus mykiss*) fry mortality syndrome in coldwater hatcheries and reared farms in Iran

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Received: July 2017

Accepted: October 2017

Abstract

An investigation was conducted in order to determine the etiological factors of Fry Mortality Syndrome (FMS) that causes a serious economical loss in rainbow trout's farms in Iran and around the world. The increased number of farms and the improvement of culture techniques and facilities in Iran had boosted the annual production of trout from 280 tonnes in 1978 to more than 30,000 tones in 2004. But unfortunately, in recent years, the rate of fry and juvenile mortalities has increased dramatically in some provinces. During 15 months, from Nov.2001 till Feb.2003, 104 tissue specimens consisting of liver, kidney, spleen, pancreas, intestine, and gills from 59 diseased fry as well as 45 infected fingerlings and suspected adults fish from Mazandaran, Tehran, Fars, Markazy, Kordestan, Kohgiluyeh and Boyerahmad provinces were collected for histopathological study. The clinical signs of the affected fry were darkening of the body, exophthalmia, ascites, erratic swimming and whirling, lethargy, gathering near the outlet of the ponds and presence of the faecal casts. Sampled tissues were fixed in 10 % buffered formalin for a minimum of 24 hours. The fixed tissues were processed in an automatic tissue processor using standard method. Processed tissues were embedded in paraffin wax and 5 µm sections were then cut using a rotary microtome. The sections were stained using H & E staining and examined under a compound microscope. Microscopic examination of the tissues revealed marked changes as follows. There were congestion and inflammation of the basal membrane of secondary lamellae, hyperplasia and fusion of secondary lamellae, and clubbing. In the kidney, congestion of blood vessels, degeneration and necrosis of hematopoietic tissue and tubules, increased melanin pigments and inflammatory cells infiltration were observed. In the liver, congestion of blood vessels of parenchyma, vacuolating changes in hepatocytes, congestion and dilation of sinusoids with the increased presence of monocytes and melanomacrophage centres (MMC) and focal necrosis were seen. Bile duct neoplasia (cholangioma) was also present in some cases. Spleen showed congestion, hemosiderosis, the increased presence of MMC and necrosis in some cases. In the pancreatic tissues, congestion, degeneration and necrosis of acinar cells and Islets of Langerhans were observed. Congestion of submucosal layer, necrosis and detachment columnar and mucous epithelial layer were also observed in the intestinal tissue. From clinical and histopathological changes seen, it was postulated that the causative agent of the trout fry mortality is likely to be a viral agent and the pathological signs were similar to IHN disease.

Keywords: Iran, Rainbow trout, Fry mortality Syndrome, Histopathology

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Introduction

In recent years, some considerable mortalities have been reported in fry and juveniles of rainbow trout in several times in Denmark (Lorenzen *et al.*, 1992), Japan (Wakubayashi *et al.*, 1991), Chile (Bustos *et al.*, 1995).

This disorder was recognized as Trout Fry Mortality Syndrome and mortality rate could reach up to 50-60% in affected fish (Lorenzen, 1991). It seemed that the syndrome is a septicemia infection and causes high economic losses in European aquaculture (Faruk *et al.*, 2002). Same disorders have been reported continuously from different ecosystems of coldwater fish in the world but were called under different names in scientific documents, such as Early Mortality Syndrome and the syndrome have special importance in North America and European countries.

From 1968 to the present, early life stage mortality has been documented in salmonids from Lakes Ontario, Michigan, but economic losses were not significant (Wolgamood *et al.*, 2005). Species exhibiting mortality include lake trout (*Salvelinus namaycush*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), steelhead (*Oncorhynchus mykiss*), and brown trout (*Salmo trutta*). Early life stage mortality varies from 1968 through 1992 and tends not to exceed 20-30% for any species.

In recent years, the rate of fry and juvenile mortality increased

dramatically in some provinces in Iran. Most mortality has occurred with unknown causative agents in different stages of fry life period (after yolk-sac absorption till before active feeding) (Fallahi, 2004).

According to annual reports of Iranian Fisheries Organization, nearly 58,345,900 fry equal to 49% of total production died due to different disease agents. Meanwhile, the mortality rate of fry and juvenile in some provinces reached up to 95% at the same time. According to statistics of Aquaculture Deputy in Iranian Fisheries Organization, 23 million fries were produced in hatcheries of Chahar Mahal Bakhtiari province in 2002, but almost 21 million fries (91.3%) in different stages of growth died before distribution to grow-out farms. Also, nearly 23 million fry were produced in Mazandaran province but about 12 million equals to 52.12% died due to diseases. It should be mentioned that above provinces provided 29.72% of total country production with total production of 4778 metric tones cold water fish annually. Soltani *et al.* (1996) reported that *Cytophaga flexibacter*-like bacterium as a causative agent in the affected coldwater farm that located beside of Haraz River in the north of Tehran. Thus, this histopathological study was conducted in order to elucidate the cause of morbidity and mortality in the cultured rainbow trout's (*Oncorhynchus mykiss*) fry in coldwater hatcheries and fish farms in Iran.

Materials and methods

Some 104 tissue specimens consisting of liver, kidney, spleen, pancreas, intestine, and gills from 59 diseased fry as well as 45 infected fingerling and suspected adult fish from Mazandaran, Tehran, Fars, Markazy, Kordestan, Kohgiluyeh and Boyerahmad provinces

were collected from November 2001 till February 2003 for histopathological studies (Table 1). All samples were collected in accordance to OIE protocol (OIE Manual of Diagnostic Tests for Aquatic Animals, 2006).

Table 1: Provinces where samples were collected for study.

No.	Farm name	Province	Date of Sampling	Code of Farm	Samples quantity (pcs)	
					Fry	Adult
1	Kelardasht Sahid Bahonar hatchery & rearing center	Mazandaran	17/10/01	BK	3	
2	Saleh Bayat sharyar	Tehran	6/11/01	CI		15
3	Saleh Bayat sharyar	Tehran	13/1/02	E		16
4	Haraz Ghezel Vana	Mazandaran	12/2/02	I	1	
5	Haraz Ghezel Vana	Mazandaran	26/2/02	J	3	
6	Haraz Ranghin Kaman	Mazandaran	26/2/02	M	3	
7	Mr.Jafari (Si Sakht)	Kohgiluyeh and Boyerahmad	26/5/02	Q	5	
8	438 cooperative Co. (Si Sakht)	As above	26/5/02	R	5	
9	Mr.Jamshidian (Si Sakht)	As above	26/5/02	S	5	
10	Mr.Amini fard (Tangheh Barayie)	As above	27/5/02	T	4	
11	Shahid Motahari Yasouj	As above	28/5/02	U1	10	
12	22 Bahman Sepidan Shiraz	Fars	29/5/02	V	10	
13	Mr.Dehghani (Sarab Bayza)	Fars	6/6/02	Ds	4	Finger-lings
14	Shahid Motahari Yasouj	Kohgiluyeh and Boyerahmad	6/11/02	U3		9
15	Mr.Hashemi (Arak)	Markazi	27/1/03	X	6	
16	Mr.H.Pezeshki (Baneh)	Kordestan	5/2/03	PK		5
Total Samples					59	45

Besides taking fish samples, other necessary information such as history and background of diseases as well as main clinical signs were also recorded. During the sampling, water temperature was recorded at less than 12°C while water quality and other environmental parameters and nutrition were within normal range.

The clinical signs of the affected fries were darkening of the body, exophthalmia, ascites, erratic swimming and whirling, lethargy, gathering near the outlet of the ponds and presence of faecal casts in the fry. Affected fish were sampled alive from the bottom of raceway and ponds with a wooden basket or suchok. Immediately after collection, a small slit in abdominal area was made on each fish before putting them into bottles containing 10% buffered formalin fixative and labelled appropriately (Fig.1). Samples from broodstock and adult were treated similarly. Some naïve fish samples were selected as control. All fixed samples were transferred into fresh fixative after 24 hours and then delivered to Aquatic Histopathology Laboratory of Tehran University



Figure 1: Collected samples in 10% buffered formalin.

The fixed tissues were processed after two months using an automatic tissue processor following standard method. After processing, tissues were embedded into paraffin wax and 5 µm sections were prepared using a rotary microtome. Finally, the sections were stained using H & E staining method and examined under a compound microscope.

Results

Clinical signs

The affected fries were dark in color, showing exophthalmia, ascites with faecal casts, erratic swimming and whirling, off-feed. The fries become lethargic as the disease progressed (Fig. 2) and swam weakly near the surface and close to edges or wall of the pond. Sometimes they gathered near the outlet.



Figure 2: Affected fry showing darkening, exophthalmia and swollen abdomen.

Histopathological changes

In this study, histopathological changes were categorized separately to kind of tissue such as kidney, liver, pancreas, spleen, gill and intestine. More than 95% of examined samples revealed

considerable lesions. Often lesions were spread in generalize forms that it could be a sign of general infection.

More details of tissue damages and histopathological findings are following as:

Kidney: There were mild to severe congestion with haematopoietic and excretory areas undergoing degeneration and necrosis, melanin pigments deposition and infiltration of inflammatory cells (Figs. 3, 4, 5).

Liver: Liver showed fatty degeneration, cloudy swelling, congestion and focal necrosis. There were sinusoids dilatation, mononuclear cells infiltration, and increased presences of melanomacrophage centres (MMC) were observed (Figs. 6,7). In some cases, neoplasia of bile duct (cholangioma) was seen (Fig. 8).

Pancreas: In some cases, severe congestion, necrosis of pancreatic acinar cells and islets of Langerhans were observed (Fig. 9).

Spleen: Spleen congestion, hemosiderosis, increased the presence of MMC and severe necrosis was important findings (Fig. 10).

Gill: Marked clubbing of gill filaments was observed due to secondary lamella hyperplasia and fusion. Some capillary congestion was also observed (Figs. 11, 12).

Intestine: Submucosal congestion, necrosis and increase the presence of eosinophilic granular cells were seen in intestinal layer (Fig. 13) and compared with normal section (Fig. 14).

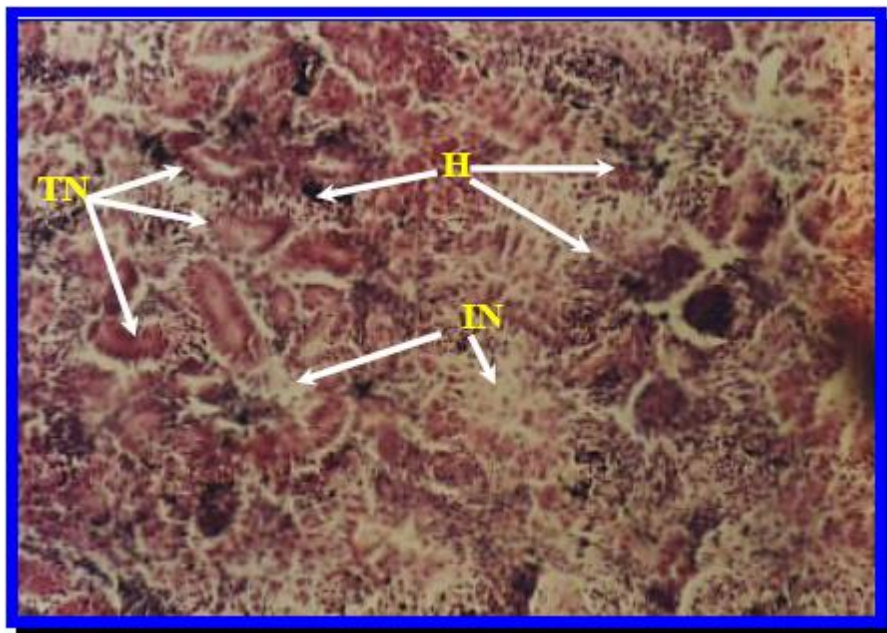


Figure 3: Marked tubular necrosis (TN), interstitial tissue degeneration (IN) and increase of hemosiderin pigments (H); (H&E, Mag. 105x).

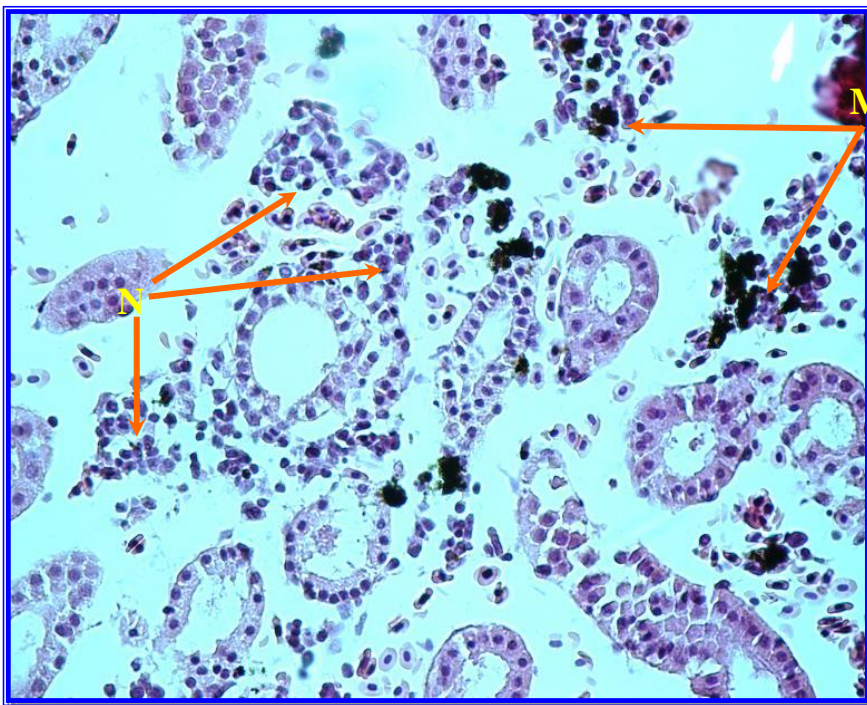


Figure 4: Oedematous degeneration and necrosis in the posterior kidney (N), Melanin deposits are normal (M); (H&E, Mag.X210).

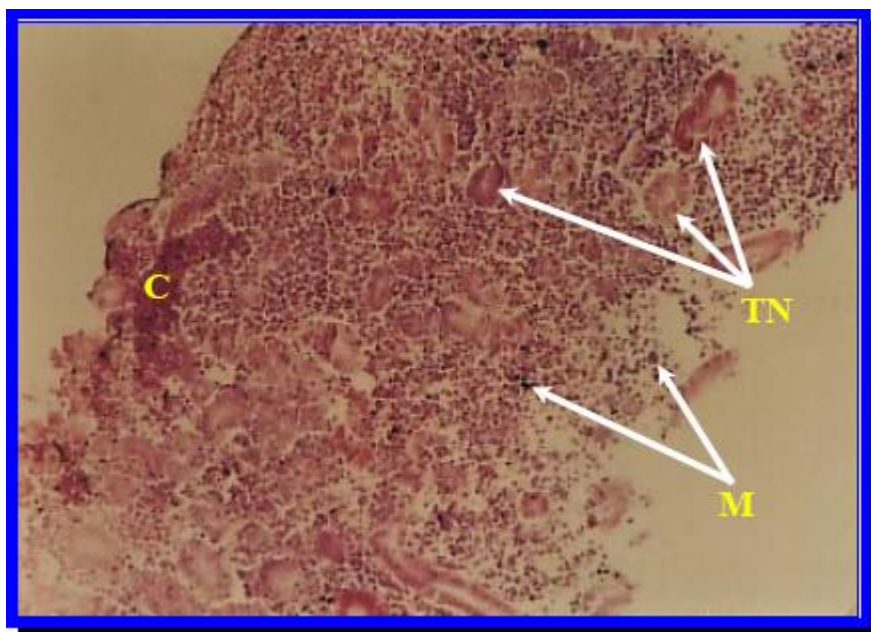


Figure 5: Congestion(C), tubular necrosis (TN), melanin pigments (M) (H&E, X105).

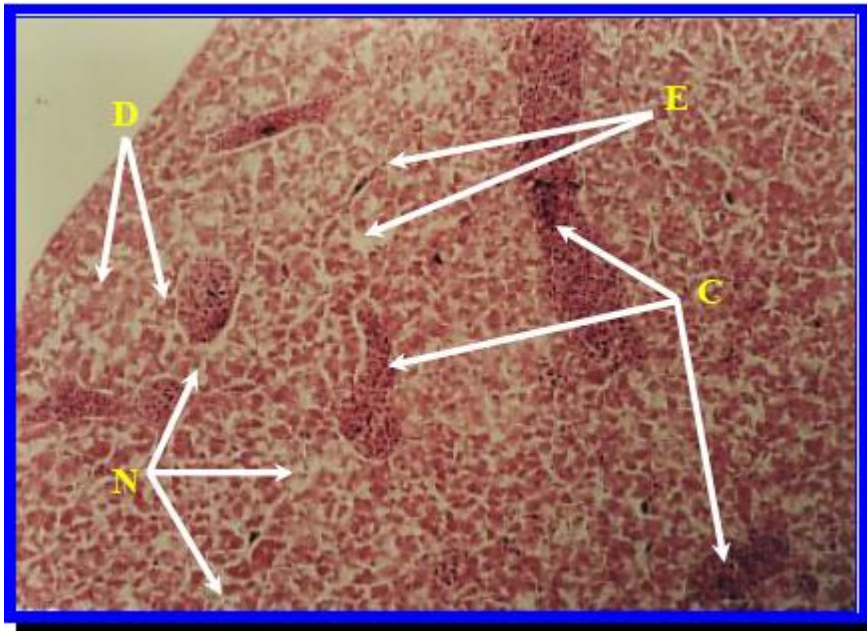


Figure 6: Severe congestion (C) in hepatic vessels, increase spaces between the hepatocytes and sinusoids dilation (E); (H&E, Mag. 105x).

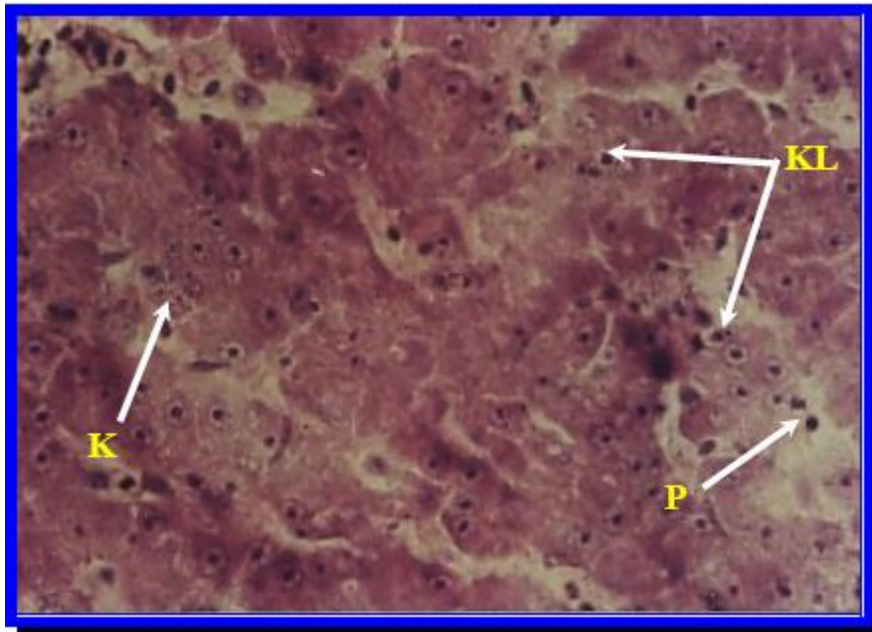


Figure 7: Stages of hepatocytes degeneration: karyorrhexis (K), karyolysis (KL) and pyknosis (P); (H&E, X420).

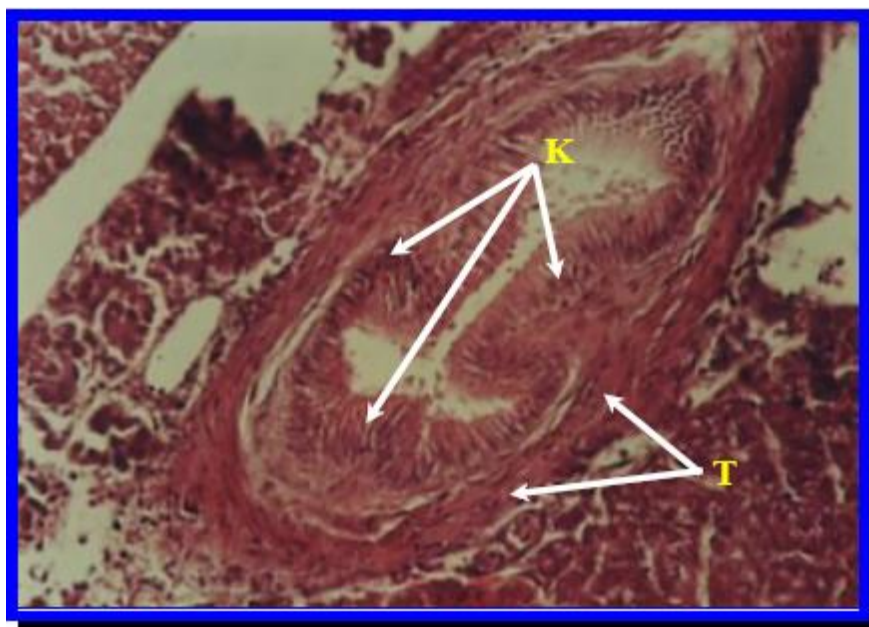


Figure 8: Bile duct neoplasia (Cholangioma), proliferation of bile duct epithelium (K) and propagation of connective tissue around of bile duct (T); (H&E, X 210).

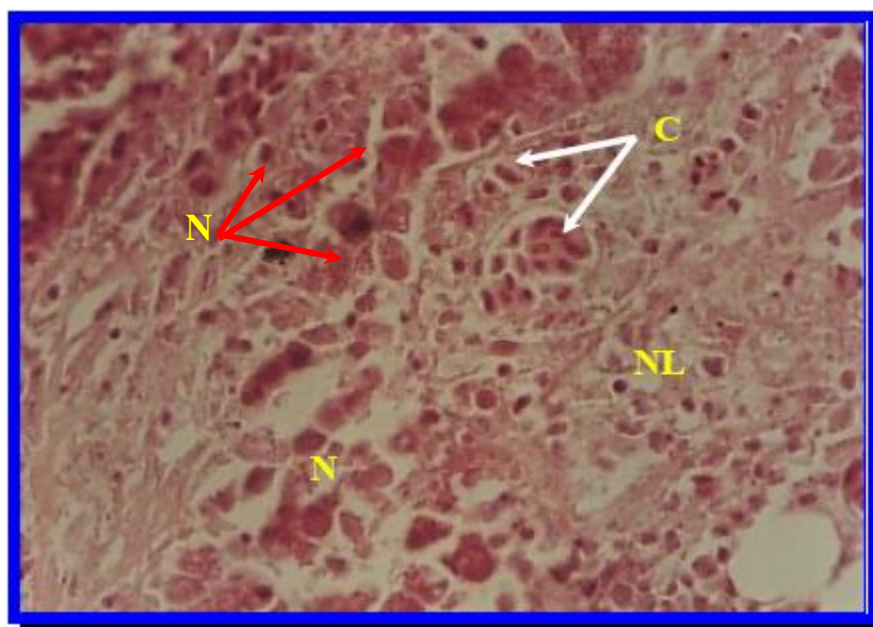


Figure 9: Necrosis of pancreatic acinar cells (N) and Islets of Langerhans (NL). Congestion (C); (H&E, Mag. 420x).

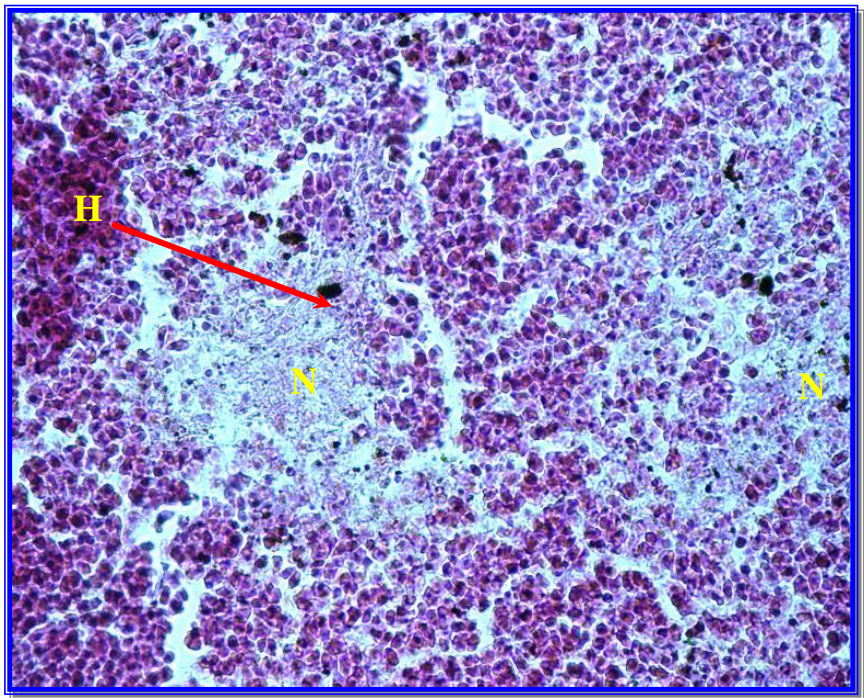


Figure 10: Spleen severe necrosis (N), hemosiderin pigments sedimentation (hemosiderosis) (H), (H&E, X210).

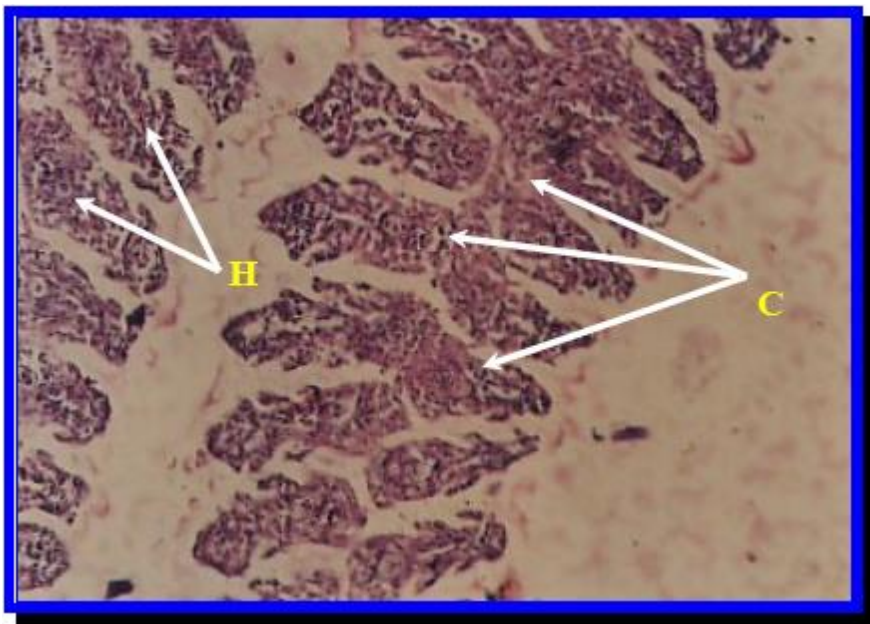


Figure 11: Gills filament showing lamella hyperplasia (H); Congestion (C) (H&E, X105).

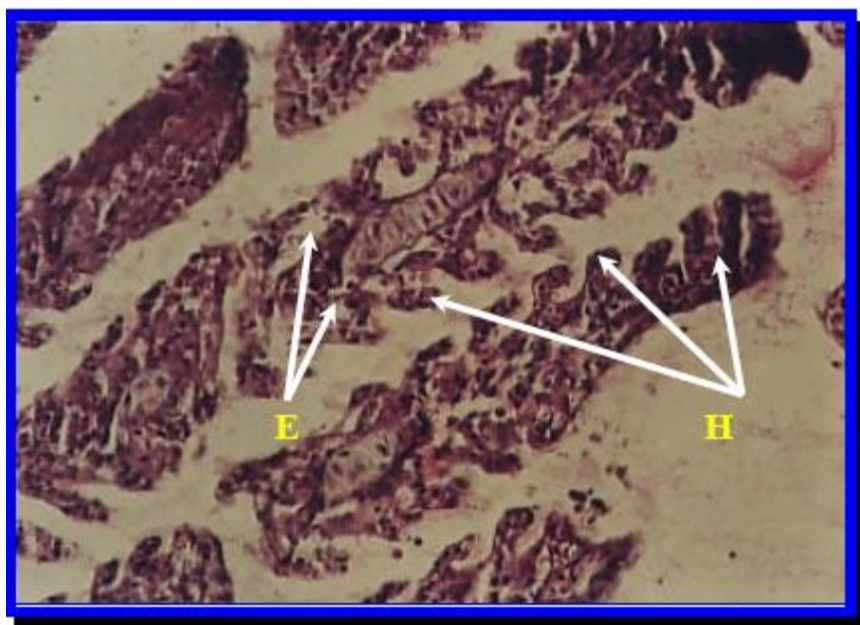


Figure 12: Hyperplastic gill lamellae undergoing necrosis. (H&E, Mag. 210x).

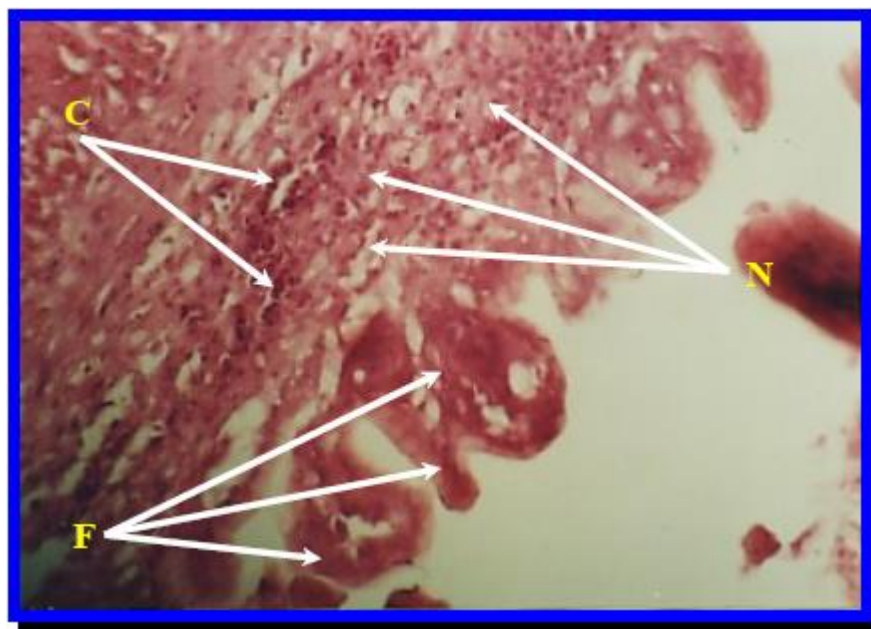


Figure 13: Submucosal congestion (C), necrosis (N), and submucosal adhesiveness (F); (H&E, X210).

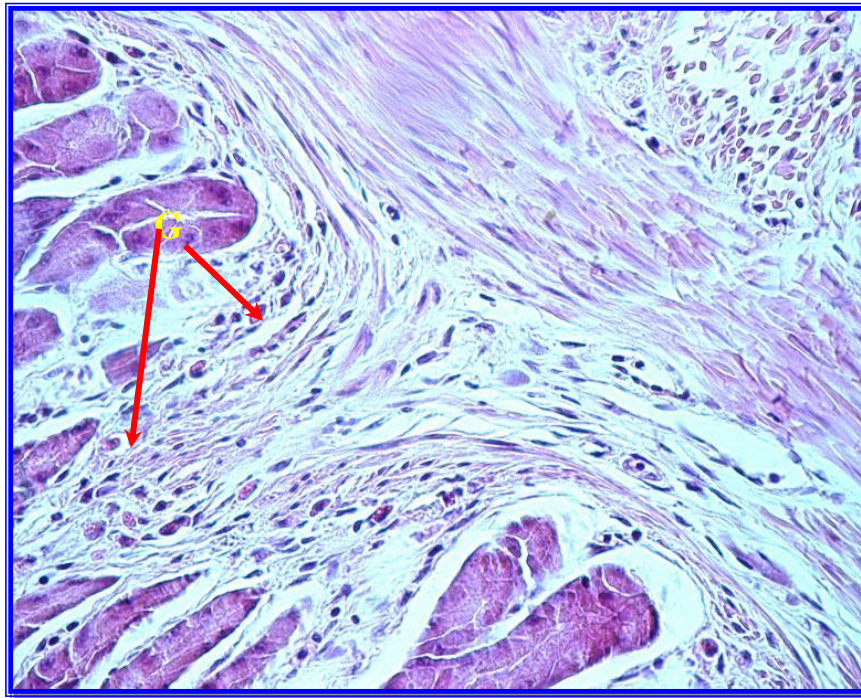


Figure 14: Eosinophilic granular cells in a submucosal layer of the intestine (EGC); (H&E, Mag. 210x).

Discussion

Clinical manifestations of abnormal swimming, lethargy, darkening of body, bilateral exophthalmia, abdominal distention with ascites and presence of the fecal casts were indicative of viral infection in coldwater hatchery and rearing farms such as infectious hematopoietic necrosis (IHN), infectious pancreatic necrosis (IPN) and viral hemorrhagic septicemia (VHS) (Roberts and Shepherd 1997; Roberts, 2001; Soltani, 2001; Mokhaye, 2002). But in the current study, some marked differences were observed such as spiral swimming behaviour. In IPN, the head of the fry pointed to the bottom of the pond and the tail pointed to the water surface (Soltani, 2001). This position was not observed in investigated fish.

While in VHS, the clinical signs may vary with the severity of infection (Yasutake, 1970; Wolf, 1988). Acute signs are typically accompanied by a rapid onset of heavy mortality. In the current study, mortality rate varied in different farms and provinces. In addition to lethargic, dark in colour and exophthalmia, the affected fish showed anaemic appearance and haemorrhages were evident in the eyes, gills, skin and at the bases of the fins. Internally, punctiform haemorrhages were evident in periocular tissues and in skeletal muscles. Liver appeared mottled and hyperemic.

In chronically infected fish, severe anaemia was noticed. Fish that were examined revealed abdominal distention without haemorrhages and any notable gross sign.

Most clinical signs in examined fish were similar to IHN such as erratic swimming, grouped near water outlet raceway or edge of rearing ponds. The presence of the faecal casts in fry was important and could be an indicator of IHN infection (Soltani, 2001; Shahsavani and Pighan, 2003).

Results of a histopathological study on collected fry revealed that vital organs such as kidney, liver, spleen, pancreas, intestine and gills had a different degree of tissue changes from mild degeneration to complete necrosis.

In many of cases stages of necrosis consist i.e. karyorrhexis, karyolysis and pyknosis could be seen. In IPN, severe and extensive necrosis in hepatopancreas was seen and affected particularly the pancreatic acinar cells and islets of Langerhans. Furthermore, congestion and haemorrhages in glomeruli, interstitial oedema and destruction of the epithelial layer of renal tubular were pathognomonic lesions. The destruction of submucosal layer leads to acute enteritis and produce fecal cast in affected fish (Soltani, 2001).

Severe necrosis in hepatopancreatic tissues, as well as changes in kidney, liver and intestine, could not pin-point to the causative agent in fry mortality syndrome in Iran. Argument, IHN and VHS agents could not produced all the lesions seen. While on the other hand, IPN as well as induced severe pancreatic cells necrosis, there is also the presence of McKnight cells in the intestinal lumen. Pancreatic tissue

rarely was affected in VHS (Shahsavani and Pighan, 2003) and intestine and gills often were normal (Mokhayer, 2002). In examined fish, the mentioned tissues showed a variety of pathological changes.

Necrosis of granular cells in a submucosal layer of the intestine is another pathognomonic sign that only occurs by IHNV (Bruno & Poppe 1996). Necrosis of granular cells was not observed in examined samples but other lesions consist of congestion, necrosis and submucosal adhesion was found.

Most histopathological changes were observed in the kidney of suspected fish and different degrees of necrosis were found in both of renal tubules and interstitial cells of a hematopoietic part of the kidney. Spleen and liver changes are similar to in IHN, VHS and IPN in affected fish. Increase in MMC presence was seen in many samples. MMC is known to be involved in defense mechanisms against various microbes (Roberts, 1975). Therefore the increase of MMC could prov of infectious agents involvement in the mortality.

Gill tissue in affected fry became hyperplastic and fused forming club-shaped. These lesions were reported in many outbreaks of IHN but it is not specific for IHN and could be originated from other non-infectious causative agents such as chemical contamination and environmental pollution.

So far there is no record and case report of high antibody titer against IHNV in gills of affected fish but gills could be as an organ target for virus localization especially in pillar filaments of secondary lamella (Soltani, 2001).

Finally, with reference to clinical signs, gross pathology and histopathological findings; it is suspected that a viral disease is the main causative agents in Trout Fry Mortality Syndrome in the coldwater hatchery and rearing farms in Iran. It is similar to IHN.

But assumption based on clinical signs, gross pathology and histopathology is not definitive. The findings are for a preliminary diagnosis. Thus more accurate and definitive techniques such as immunofluorescent antibody test (FAT) could be used on suspected tissue samples. For more sensitivity and specificity, another diagnosis approach such as ELISA, IHC and molecular biology study (RT-PCR, real-time PCR) could be employed.

OIE manual recommended that virus isolation for new case report could be more significant. After virus isolation, Koch's postulate test should be carried out to evaluate the isolated virus. Serological methods such as neutralization test (NT) or FAT and enzyme-linked immunosorbent assay (ELISA) will confirm the agent. (OIE, 2006).

Acknowledgements

The authors would like to thank the Iranian Fisheries Research Institute (IFRO) for financial support in this study. We also thank Mr. H. Bagheri and Mr. Saydanlou, staff of Aquatic Animal Health & Diseases Lab. in Veterinary Faculty of Tehran University.

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