

Histopathological Studies in Experimentally Infected Koi Carp (*Cyprinus carpio koi*) with Koi Herpesvirus in Japan

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Abstract: Many carp and koi farms have been afflicted by a disease with a high mortality rate, resulted in severe financial losses. This disease, caused by a koi herpesvirus (KHV), is highly contagious and its mortality and morbidity are restricted to koi and common carp populations. This study was performed to elucidate the histopathological effects of KHV, after challenge into the gills of fish with 100 µL IKCO3 suspension, on some internal organs at various times. In this report, KHV was confirmed in tissues by electron microscopy and KHV specific (PCR). The histological examination revealed glomerulonephritis associated with periglomerular fibrosis and hyperplastic changes in renal tubules. There are severe gill lesions evidenced by loss of lamellae in some fish, besides fusion in the lamellae due to hyperplasia in the respiratory epithelial cells, other showed hemorrhagic patches on the tips of lamellae. It could be concluded that KHV not only caused necrosis gills and interstitial nephritis but also induces hyperplastic lesions in kidneys, stomach and intestine.

Key words: Electron microscopy • Histopathology • Koi herpesvirus (KHV) • Koi carp (*Cyprinus carpio koi*) • KF-1 • PCR assay

INTRODUCTION

Koi herpesvirus (KHV) is a contagious disease encountered in several fish farms (common carp *Cyprinus carpio carpio* and koi carp *Cyprinus carpio koi*) in Israel during spring 1998, following outbreaks in U.S., United Kingdom, Germany and Indonesia [1-3]. In European, African and Asian countries, KHV infection becomes widely spread and causes significant losses in the common carp *Cyprinus carpio* [2, 4, 5]. KHV can transmit via the water, fecal material, sediments and infected fish [6, 7]. The infection usually occurs through the gill [7], gut [8] and kidneys [2]. Based on its pathogenic effect in fish, it has also been termed Carp Interstitial Nephritis and Gill Necrosis virus (CNGV) [9, 10]. Isolated and identified a Herpes-like DNA virus from the infected carp was similar in structure and morphology to Cyprinid herpesvirus (CHV) [2]. It has been recognized as the agent associated with a serious and lethal disease among koi and common carp of all ages. It causes papillomatous skin growths in older fish [2, 11, 12]. The harvested virus from Koi fin cell (KFC) induces the same natural disease in fish and causes mortality of 75-100% upon inoculation of naïve koi and common carp [10, 13]. Previous study established a novel

KHV challenge method (the per-gill infection method) with live KHV for experimental infection and reported the histopathological changes of KHV infected cells [14, 15]. The aim of the present study was to study the pathological changes in the tissue of the most organs induced by challenge of KHV in koi carp (*Cyprinus carpio koi*).

MATERIALS AND METHODS

Viruses: KHV isolate (IKCO3 from Ibaraki Prefecture that was kindly donated by Dr. H. Fukuda, Tokyo University of Marine Science and Technology) was provided. The KHV isolate was cultured using KF-1 cells donated by Dr. R. P. Hedrick (University of California Davis) according to Hedrick *et al.* [2].

Cytopathic effects of the KHV: Koi herpesvirus (KHV) was cultured in koi fin (KF-1) cells and keep in incubator at 20°C for 5 day. The isolated virus produced plaques on KF-1 cells at 4 to 5 days post-challenge. The infected cells increased daily and large cytoplasmic vacuolations were seen in several cells (Fig. 1).

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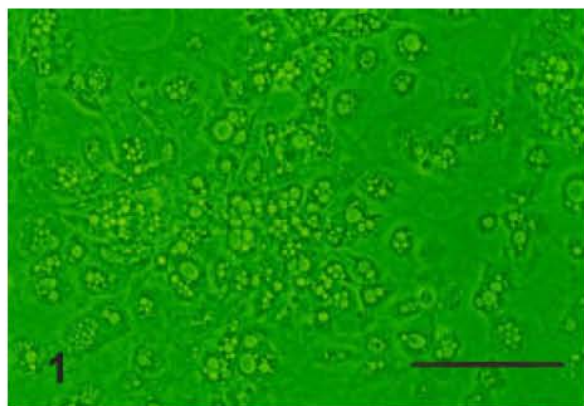


Fig. 1: The isolated virus produced plaques on KF-1 at 4 to 5 days post-challenge. Occurrence of large cytoplasmic vacuolations within the infected cells. Bar = 50, μm

Experimental Fish: Koi carp (*Cyprinus carpio koi*) (n= 20) (mean body weight 30 g) were obtained from a private koi farm in Niigata Prefecture. The fish were kept in tanks with water-circulation and filtration system at 24°C.

Experimental Design: The present study was carried out on a total number of 20 fish which are divided into two groups:

Treated group (n= 10) was challenged with KHV. Carp were anesthetized by ethyl carbonate 1.5 % and inoculated with 100 μL IKCO3 suspension into their gills (50 μL to each side of gills) in air (23°C). IKCO3 was inoculated at a dose $10^{2.0}$ TCID₅₀/100 μL^{-1} (n=10 of koi carp). After treatment, fish were wrapped with wet papers and kept in air for 5 min to complete KHV adhesion to gill cells at air- conditioned room temperature (23°C) and then returned to the tanks. The control group (n= 10 of koi carp) was treated with saline instead of KHV. KHV-inoculated fish were fed on a pellet diet and observed for clinical signs and mortalities at 22°C for 21 day.

Histopathology and Electron Microscopy: Moribund fish were removed from the tank and pieces of gills, liver, spleen, kidneys, stomach and intestine were fixed in bouin's fluid and processed for histopathological examination. Tissue sections were stained with hematoxylin and eosin (H& E). In addition, Pieces of gills, kidneys were fixed in 70% karnovsky's solution, post-fixed in 1% osmium tetroxide (OsO_4) and processed for electron microscopy. Samples from gills and kidneys were stored at -80°C for PCR assay [5].

Polymerase Chain Reaction (PCR): DNA extraction was carried out from gill and kidney specimen (5-10 mg) using phenol-ethanol method. Then 1ul of the extracted DNA was used to amplify 290bp (500bp) fragment targeted by SphI-5F (5'-GACACCACATCTGCAAGGAG -3) forward primer and SphI-5R (5'-GACACATGTTACAATGGTGGC-3) reverse primer. The amplification program consists of a denaturation step at 94 °C for 1 min, annealing at 55°C for 2 min, extension at 72 °C for 3 min for 30 cycles and a final extension step at 72°C for 7 min, as previously described [5, 16]. The PCR products were electrophoresis in agarose gel (1.5%) visualized by UV transillumination and photographed.

RESULTS

Clinical Signs and Gross Appearance in the Treated Group: The diseased fish were first recorded at 5 d post-challenge. They floated on the water surface of the tank and showed shaking and twitching behavior. Many moribund or dead fish were observed (3, 3, 2, 1, 1 fish dead at 6, 7, 11, 16 and 17 days post-challenge, respectively). Most of the diseased fish displayed necrotic changes and hemorrhagic patches on gills and enlargement in kidneys. None of the saline-treated fish became moribund and all were PCR-negative for KHV.

PCR Results: The virus infected gill and kidney samples were confirmed by the detection of 290 bp (or 500bp) amplified fragment of the KHV as shown in Fig. (1a). The IKCO3 DNA was used as a positive control.

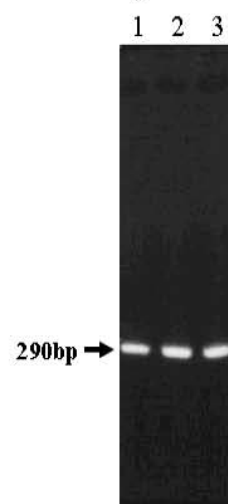
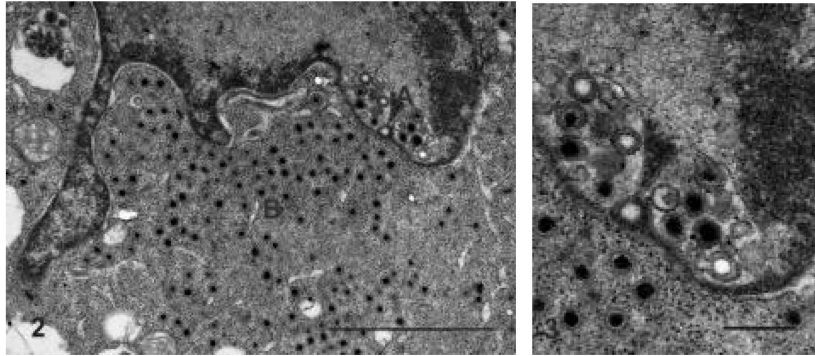
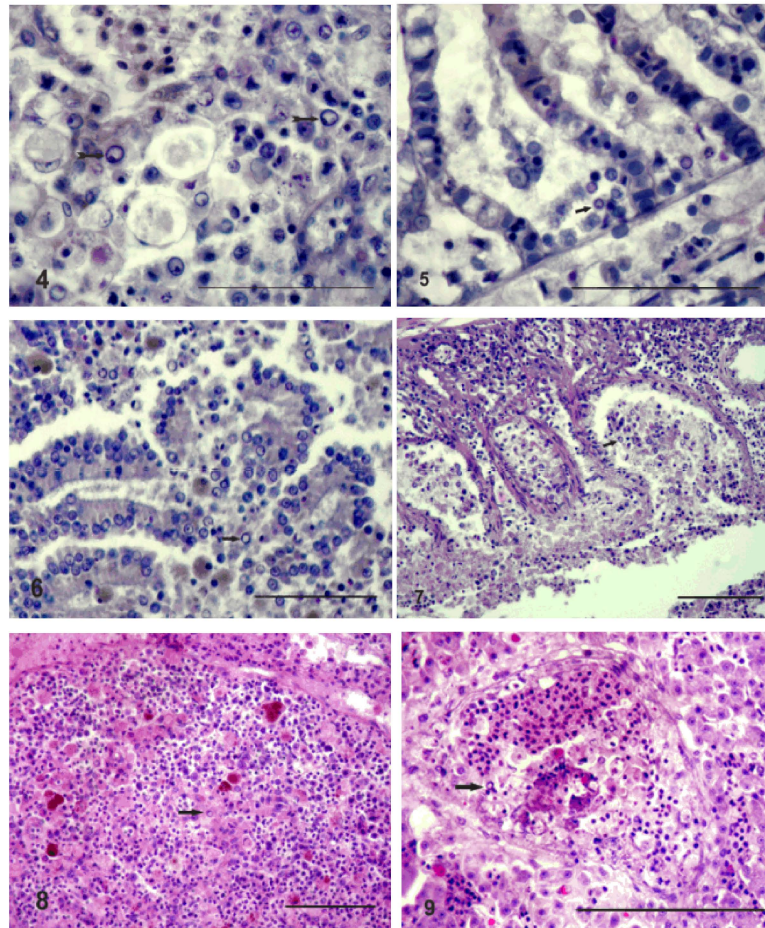


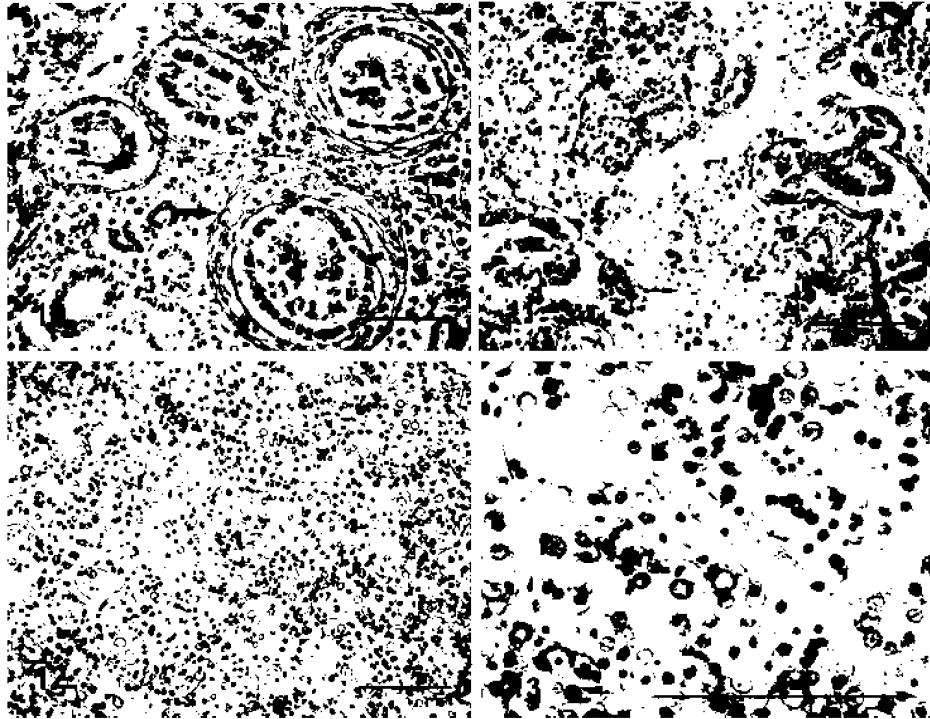
Fig. 1a: PCR results of the gill and kidney samples from infected fish with KHV
Lan 1: Positive KHV (IKCO3)
Lan 2: Gill sample Lan 3: Kidney sample



Figs. 2,3: Electron micrographs of KHV-infected respiratory epithelial cells in gill showing, (1) the tegumentation of nucleocapsids takes place within the inclusion body (A) and membranes delimited structures, besides mature nucleocapsid with electron-dense cores (enveloped virions) were embedded into the cytoplasm (B). Scale bar = 1000 nm (2) high magnification of figure 2 show the inclusions containing assembly of mature and immature virions. Scale bar = 200 nm



Figs. 4-9: The KHV in all organs showing, (4) kidneys exhibited marginal deposition of heterochromatin in the inner nuclear membrane of the hoemopoitic cells (arrow). HE. Bar, 100 µm. (5) gills, the respiratory cells in the gills appeared swollen and vacuolated with degenerated nucleus (arrows) HE. Bar, 100 µm, (6& 7) The KHV induced the same lesions in the hyperplastic cells of the stomach and intestine (arrow) HE. Bar, 100, 50 µm, (8) spleen showed splenocytes with hyperchromatic nuclei (arrow). HE. Bar, 50 µm, (9) liver, many hoemopoitic cells with hyperchromatic borders among RBCs inside the hepatic blood vessels (arrow). HE. Bar, 100 µm.



Figs. 10-13: Kidneys showing, (10) periglomerular fibrosis, manifested by bundles of fibrous tissues surrounded the destructed glomeruli formed nodules (arrow), (11) interstitial nephritis with loss of haemopoietic cells, tubular epithelial cells with pyknotic nuclei, (12 & 13) the caudal areas are closely packed with the tubules with broken membranes, the tubular cells appeared different in size, large, vesicular, hyperchromatic nucleus and dark eosinophilic cytoplasm, besides necrosis in some cells. HE. Bar, 50, 100 μ m

Detection of KHV in Tissues by Electron Microscopy:

The infected respiratory epithelial cells of the gill lamellae and kidneys cells with KHV were examined by electron microscopy. In the KHV infected cells of gills at 16 d post-challenge, mature nucleocapsid with electron-dense cores (enveloped virions) were embedded into the cytoplasm. Assembly of both immature capsids without a core or nucleocapsids and mature nucleocapsid embedded in the nucleus which displayed deposition of heterochromatin on the inner nuclear membrane. Many tegumented particles embedded within membrane-delimited structure, inclusion bodies inside the nucleus. Other cells showed many virions, with dark intranuclear inclusion body, one tegumented particle is in a membrane-delimited structure, besides many enveloped virions budded into cytoplasm. The tegumentation of nucleocapsids takes place within the inclusion body and membranes delimited structures (Figs. 2 & 3).

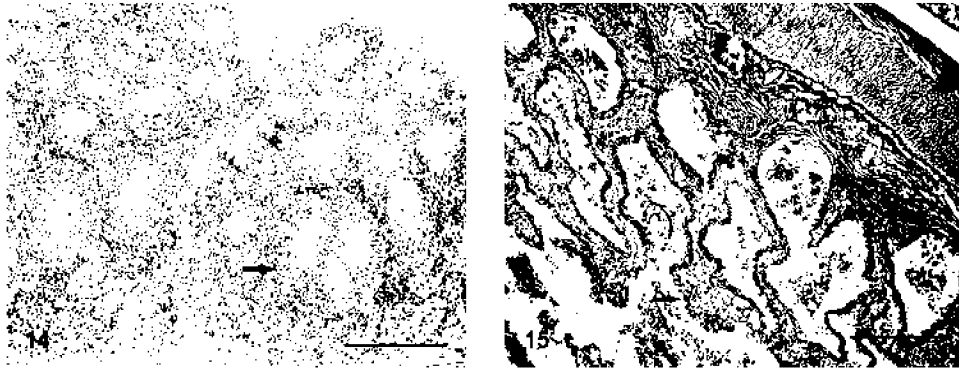
Histopathological Findings

Detection of KHV in the Tissues: The haemopoietic cells in the kidneys were depleted and necrosed. The epithelial cells lining the renal tubules exhibited nuclear marginal

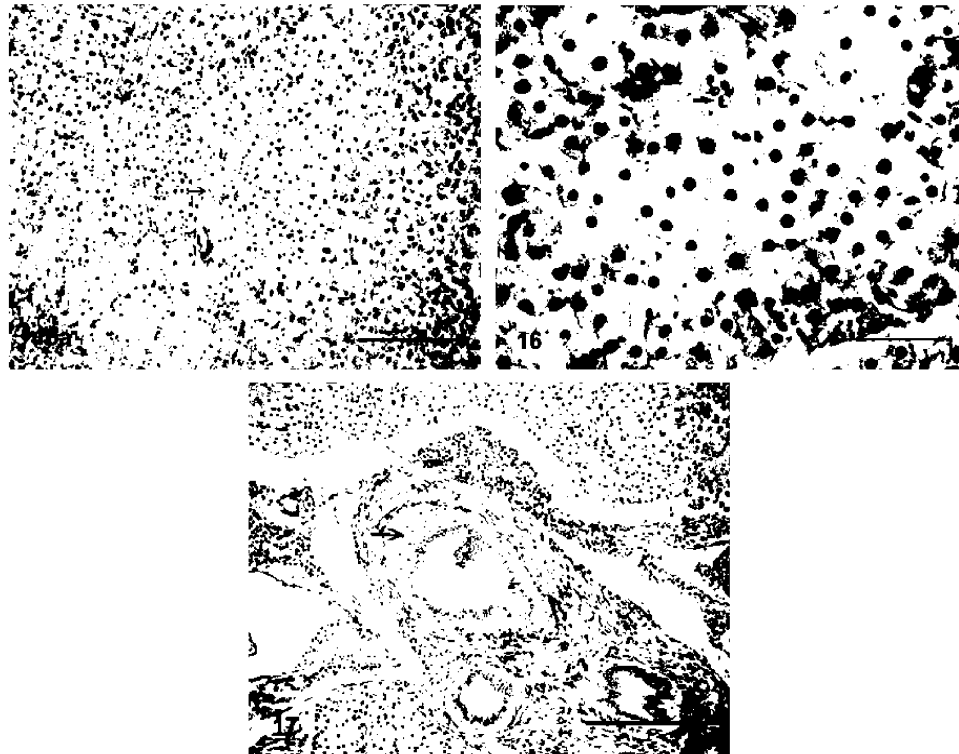
hyperchromatosis caused by deposition of heterochromatin on the inner nuclear membrane (Fig. 4). The respiratory cells of the gills appeared swollen and vacuolated with degenerated nucleus (Fig. 5). KHV induced the same lesions in the hyperplastic cells of the stomach and intestine (Figs. 6 & 7). In spleen, splenocytes exhibited marginal hyperchromatic nuclei (Fig. 8). In the liver, hepatocytes and many haemopoietic cells with hyperchromatic borders among RBCs inside the hepatic blood vessels were seen (Fig. 9).

The Pathological Lesions Induced by KHV in the Tissues

In kidneys: The haemopoietic cells were depleted in all dead fish. Many of the cells were necrosed and had a vacuolation (marginated chromatin) in the nucleus. Fish die at 6 & 7 d post inoculation (PI) displayed necrosis of the renal tubules with intranuclear inclusion body. The kidneys at 11 d PI exhibited hyperplasia of the epithelia cells of renal tubules and sloughing inside the lumen. Moreover, chronic glomerulonephritis associated with periglomerular fibrosis was noticed. It is manifested by bundles of fibrous tissues surrounded the destructed glomerulus forming nodules inside the renal parenchyma.



Figs. 14,15: Stomach & intestine showing, (14) Hyperplasia in epithelium lining of the gastric gland (arrow) causing occlusion of the lumen HE. Bar, 25 μ m, (15) hyperplasia in the intestinal cells with sloughing inside the lumen (arrow). HE. Bar, 50 μ m

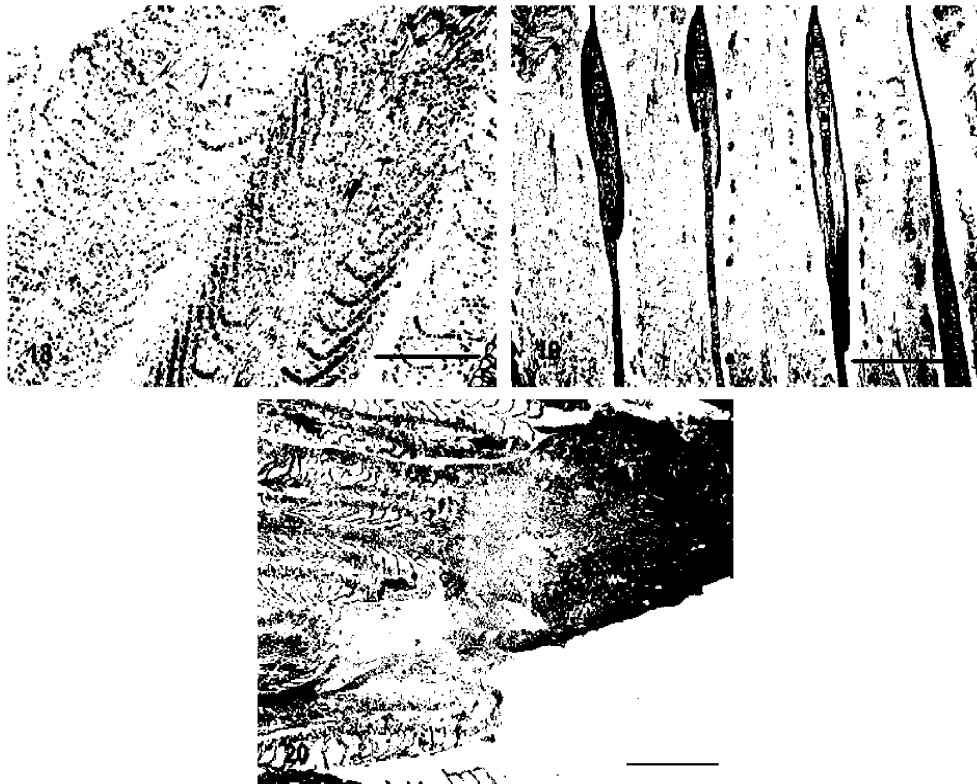


Figs. 16a,16,17: Liver showing, (16a) necrosis in the hepatocytes. HE. Bar, 25 μ m, (16) high magnification of (Fig. 16a) to show the necrotic cells HE. Bar, 50 μ m, (17) Proliferation in the billiary epithelial cells. HE. Bar, 25 μ m

Interstitial nephritis with loss of hoemopoitic cells and pyknosis of tubular epithelial cells were observed. Some necrotic cells showed vacuolation with intranuclear inclusion bodies (Figs. 10& 11). On the other hand, the caudal part of kidneys is closely packed with the tubules, noted variation in its cells size with intact membrane. The tubular cells showed large, vesicular, hyperchromatic nucleus and dark eosinophilic cytoplasm, besides necrosis and degenerations (Figs. 12& 13). Some tubules

showed broken membranes and the epithelial cells revealed prominent central heterochromatin, other infected cells showed hypertrophied spherical nuclei with margined heterochromatin.

Stomach: Inclusion bodies were seen in most epithelial cells. Hyperplasia in the epithelium lining of gastric gland causing occlusion in the lumen in dead fish 6 d & 7 PI (Fig. 14).



Figs. 18,19,20: Gills showing, (18) fusion in the lamella, (19) severe destruction in all lamellae, (20) focal areas of hemorrhages on the tip of lamellae. HE. All Bar, 50 μ m

The Intestine: Dead fish at 7 d PI p.i. showed hyperplasia in the intestinal villus formed cystic papillary projections inside the lumen. Most of the epithelial cells were lost and sloughed inside the lumen, besides necrosis with loss and irregularity in smooth muscle fibers in the lamina propria forming zigzag-like appearance (Fig. 15). In fish dead at 11 d PI the sloughed cells with intranuclear inclusion bodies and cellular debris were observed within the lumen.

The Liver: displayed necrosis in the hepatocytes with intranuclear inclusion bodies, besides vacuolations were noticed (Figs. 16a & 16). Proliferation in the columnar epithelial cells of biliary duct was detected (Fig. 17).

Gill: The gill in the dead fish at 6d post-challenge showed hyperplasia in the respiratory cells resulting in fusion in the lamella, some cells had intranuclear inclusion bodies with congestion in the blood vessels (Fig. 18). Other showed severe destruction in all lamellae (Fig. 19) in dead fish at 6-11 d post-challenged. Focal areas of hemorrhages were observed on the tip of lamellae in dead fish at 7d post-challenge (Fig. 20).

DISCUSSION

Koi herpesvirus infection is currently of major concern to common carp and koi carp breeders in the whole world. The disease causes severe mortalities in carp of all ages and is spreading rapidly across the globe. The present study is important not only to test and confirm the presence of KHV infection but also to describe the microscopic lesions resulted from this infection and to determine the severity of this virus on the nature of some organs. Previous study established novel per-gill infection [15]. As same as methods used in our study to stimulate a natural route of infection, the KHV (IKCO3) was cultured using KF-1 cells, 100 μ L IKCO3 suspension, (at a dose $10^{2.0}$ TCID₅₀/100 μ L⁻¹) was inoculated into their gills. The viral agent, designated as koi herpesvirus (KHV), was isolated and propagated in koi-fin (KF-1) cells [2, 11, 12, 15]. In our work, the mortalities of the inoculated fish showed among the 6th to 17th day PI, while previous study detected that most fishes infected with (4×10^3 TCID₅₀/ml) in little aquaria with water died within 24- 48 h after the first symptoms appeared [17]. The external signs seen in the moribund and dead fish in our work were necrotic changes and hemorrhagic patches on gills and

enlargement in kidneys. Previous studies displayed swollen and necrotic gill filaments, excessive mucus production or discolored patches on the skin, besides enlargement in the kidney and spleen [2, 18]. Other study, observed gill necrosis, sloughing of the epidermis and the black patches of skin discoloration in the experimental fish infected with (4×10^3 TCID₅₀/ml) in little aquaria with water [17]. Gray *et al.* [16] showed that the most consistent sign of the disease is discoloration, increased respiratory frequency while, Oh *et al.* [19] displayed swollen, pale, patchy gills and skin lesions. The results of our work confirmed the presence of KHV infections in Koi Carp (*Cyprinus carpio koi*) in Japan by PCR analysis, Electron microscope and histopathological examination. By electron microscopy, the present work revealed mature and immature virions embedded in nucleus and enveloped virions entered the cytoplasm of the KHV infected cells. Ultrastructure of koi herpesvirus reported that viral DNA is contained in the inner core of KHV and surrounded by capsid which is encompassed by a tegument layer (envelope), which incorporates glycoprotein spikes that are important for attachment and entry into host cells, hence for infectivity [15, 17]. Our work agree with previous studies, they mentioned the pathogenesis of the diseases and described the ultrastructure examination of KHV, virions are composed of an inner capsid with icosadeltahedron symmetry of approximately 100 - 110 nm in diameter and detection of KHV-specific DNA by PCR technique [15- 21]. Based on the morphology and size of the virus and the sequential development in the host cell nucleus, the virus was designated KHV for koi herpesvirus [2]. The same agent was isolated subsequently from koi and common carp by Ronen *et al.* [9]. Identification of KHV was detected in most tissues microscopically, the haemopoietic cells and tubular epithelial cells in kidneys were necrosed and their nucleus exhibited marginal hyperchromatosis. Hepatocytes, splenocytes and gastric epithelial cells infected with KHV showed inclusion bodies with marginal or central chromatin. The gill is one of the majority tissues influenced with KHV in the present study. Histopathologically, chronic glomerulonephritis was evident in our work. Moreover, the caudal part of kidneys was closely packed with renal tubules, the tubular cells noted variation in its size with vesicular, hyperchromatic nucleus and dark eosinophilic with broken membranes in some tubules. The hyperplastic lesions in the kidneys of our work, is the first record in infected fish by KHV but previous authors described skin tumors in adult common carp due to cyprinid herpesvirus (CHV) [11, 16]. Previous

study revealed necrosis of parenchyma cells and numerous macrophages with ingested cellular debris of the liver, spleen, kidney and gastrointestinal tract [2]. In the present study, the occurrence of hyperplasia of epithelium cell lining of the gastric gland with occlusion indicated that the virions that have propagated within the respiratory epithelial cells entered the capillaries to cause systemic dissemination via blood stream [15]. Based on the detection of CNGV in gill mucus and lamellae [10, 21], the virus infects the fish via the gills, replicates there, induces mucosal sloughing and necrosis and is then shed into the water. From the gills, the virus can be rapidly transferred to the kidneys, where it resides in white blood cells and induces severe interstitial nephritis. Localization of the virus within white blood cells raises the intriguing possibility that the virus is rapidly transferred to the viscera via infected white blood cells and then multiplies in the epithelial cells of the kidney and intestine. The virus is released into the water either through shedding or together with the sloughed epithelial and inflammatory cells resulting from severe local inflammation. However, other authors showed that large amounts of viral DNA were found in the gut early after infection [21] and clusters of virus particles were detected by electron microscopy in the intestinal system [13]. Thus, the possibility that the virus penetrates the fish body through the digestive system should also be considered.

It could be concluded that KHV not only caused necrosis gills and interstitial nephritis but also induces hyperplastic lesions in kidneys, stomach and intestine.

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