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Short Communication

A Histopathology Study of Caspian Seal (*Pusa caspica*) (Phocidae, Mammalia) Liver Infected with Trematode, *Pseudamphistomum truncatum* (Rudolphi, 1819) (Opisthorchidae, Trematoda)

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Abstract

Background: Main objective of this study was to investigate the invasive activity of the liver fluke, *Pseudamphistomom truncatum* against the Caspian seal (*Pusa caspica*) and was exemplified at the gross, light microscopy (LM) and electron microscopy (EM) levels.

***Methods*:** The study was done on a freshly dead Caspian Seal in the southern coast of Caspian Sea. The checked Caspian seal probably being died of canine distemper virus and was found host to numerous parasites of four helminth species.

***Results*:** *P. truncatum* caused edematous foci on the surface of the liver with prominent fluid accumulation. Sections of the liver viewed with LM had multiple necrotic areas with extensive hemorrhaging and disorganized hepatic lobules. Granulocytes and invasion of connective tissue were prominent. Whole worms were visible with invasive pathways through the host tissue. Damage to both hepatic ducts and blood vessels were prominent. At the EM level, organelles within the impacted hepatocytes were disorganized as exemplified by the cristae of the mitochondria and the endoplasmic reticulum. Parasite eggs were scattered throughout the tissue.

***Conclusion*:** It was shown that this trematode can be very pathogenic to Caspian Seal and as this only mammal of Caspian Sea is an endangered species; this needs more investigation toward control or possible treatment of this helminth.

Introduction

Pusa caspica is one of the smallest members of the earless seal family (Phocidae) and unique in that it is found exclusively in the brackish Caspian Sea and endemic to it. It is distributed throughout the Caspian Sea from avant-deltas of the Volga and Ural Rivers to the Iranian coasts. Although there are reports of animals occurring sporadically in the Volga River as far as Volgograd and 200 km upstream along the Ural River (1, 2). However in recent years there has been a decrease in their population –from more than 400000 in 1967 to 100000 in 2010 (3) due to different causes like hunting, pollution and infection diseases (4).

As this mammal is an endangered animal (5), knowing about its parasites and pathology of each parasite, can eventually lead to improve conservation programs.

There are several publications about the parasites of the Caspian seal reporting different parasites in this host (6, 7) but very little is known about histopathology of these parasites, and as this seal species is the only mammal in Caspian Sea, it can act as definitive host for several of important parasites occurring in the fishes of Caspian Sea.

Pseudamphistomum truncatum is a common liver fluke found in many carnivorous hosts (8-10). Pathology of this trematode has been studied in several other carnivores like Eurasian Otter (11), and Mink (10, 12).

The objective of this paper is to describe the pathology at three levels caused by this trematode. The liver tissue of the Caspian seal and the digenes inside were checked in gross and then observed with light (LM), Scanning and Transmission Electron Microscopy (SEM and TEM) whereby the pathological process could be described.

Materials and Methods

A Caspian seal (*P. caspica*) that was found freshly dead (Fig. 1) on the southern shore of the Caspian Sea near Ramsar City, Mazandaran Province, Northern Iran (36°55´N, 50°40´E) on April 29, 2009 was examined for parasites by the second author (AH). Cause of death was probably Canine Distemper Virus (CDV) which has been observed previously (13, 14).

After necropsy internal organs was carefully checked and representative samples were taken from each organ and placed in labeled vials containing 10% buffered formalin for further studies including histopathology and observations with the light and electron microscopes. Parasites found in the organs were isolated and relaxed, and then placed in labeled vials containing 70% ethyl alcohol. Numerous trematodes were found in the biliary tract of the liver that was also prepared for future research. Identification of parasites was based on available key references (15-17).

Light Microscopy

For the infected liver tissue, several sections were removed and the infected tissue was fixed in 10% buffered formalin for 48 hours and then stored in 70% ethyl alcohol for shipment to the laboratory. The host tissue was processed and blocked in paraffin using a vacuum automated tissue processor. Standard methods for solution changes were used (18, 19). The paraffin blocked tissue was sectioned at 4-6 um (microns), placed on glass slides and stained with hematoxylin and eosin (H & E), using an automatic staining station. The prepared slides were reviewed with an inverted compound light microscope LSM, Axiovert 135, (Carl Zeiss, Thornwood, New York) with laser illumination. Representative pictures were taken at varying magnifications. Records of observations were stored on a USB memory disk.

Scanning Electron Microscopy

For SEM studies, specimens previously fixed in 70% ethanol were placed in critical

point drying baskets and dehydrated using an ethanol series of 95% and 100% for at least 10 minutes per soak followed by critical point drying (20). Samples were mounted on SEM sample mounts, gold coated and observed with a scanning electron microscope (XL30 ESEMFEG; FEI, Hillsboro, Oregon). Digital images of the structures were obtained using digital imaging software attached to a computer.

Transmission Electron Microscopy

Ten samples of the infected organs and the parasite (*P. truncatum*) previously fixed in 10% buffered formalin and 70% ethyl alcohol were dehydrated in an ascending series of ethanol solutions. These samples had been stored in 70% ethyl (ethanol) alcohol. Samples were then rehydrated for post-fixation in 1% buffered osmium tetroxide and then dehydrated in an ascending series of ethanol, followed by two changes of 100% acetone. Specimens were then embedded in Spurr's resin and sectioned with a diamond knife, using an automated ultra microtome to a thickness of 80 to 100nm. After post staining with Reynolds lead citrate and 5% urinal acetate in 50% ethanol, sections were examined in an FEL Techani T-12, High Resolution TEM (FEI Company, Hillsboro, Oregon). Images at varying magnifications were taken with a digital camera using digital imaging software attached to a computer.

Results

Four parasites species were found in the examined seal including the liver trematode, *Pseudamphistomum truncatum*, which we described in a previous article using SEM (21) as well as an acanthocephalan, *Corynosoma strumosum* (22), *Anisakis* sp and *Eustrongylides* sp. (not reported yet).

Figures 1, 2, 3 represent the dead Caspian seal (*Pusa caspica*) (Fig. 1) and the internal organs (Fig. 2, 3). The seal probably had died from a viral infection (Canine Distemper Virus). Figure 1 represents the ventral surface of the deceased Caspian seal. Figure 2 and 3 depict the internal viscera following dissection of the seal showing the various organs. Each organ was carefully removed and observed. The liver had several edematous foci, which are grayish in color depicting trauma and fluid accumulation in the outer connective tissue. Other major organs were visible but were not included in this study. Sections of liver were labeled and placed in vials containing 10% formalin for future study. There were multifocal necrotic areas in the hepatic tissue due to parasite invasion and the viral infection.

Discussion

SEM: Figures 4, 5, and 6 are the results of the SEM scans for *P. truncatum*. We were unable to scan infected liver tissue from the Caspian seal with SEM. Figure 4 is the whole mount of the trematode showing the lancelot shape and the prominent suckers. Figure 5 is the ventral sucker and Figure 6 is the blunt posterior end of the fluke. We have published an article (21) pertaining to the SEM ultrastructure of *P. truncatum*.

Light Microscopy: Figures 7 to 24 represent the results of the histopathological examination of *P. truncatum* and its invasive effect on the liver of *P. caspica*. Figure 7 shows the route of the trematode through a hepatic lobule displacing the hepatocytes. Nucleated blood cells are visible as well as the canals between the hepatocytes. Figure 8 is a section through a normal hepatic lobule showing hepatic ducts and canals leading to the bile ducts. A hepatic blood vessel is visible on the slide, which is lined with an intact endothelium (Fig. 8). Surrounding the hepatic lobule is a network of connective tissue. Blood cells are visible flowing through the vessels. The invading fluke is visible with a section depicted by figure 9. Necrotic tissue and hemorrhaging are extensive next to the parasite.

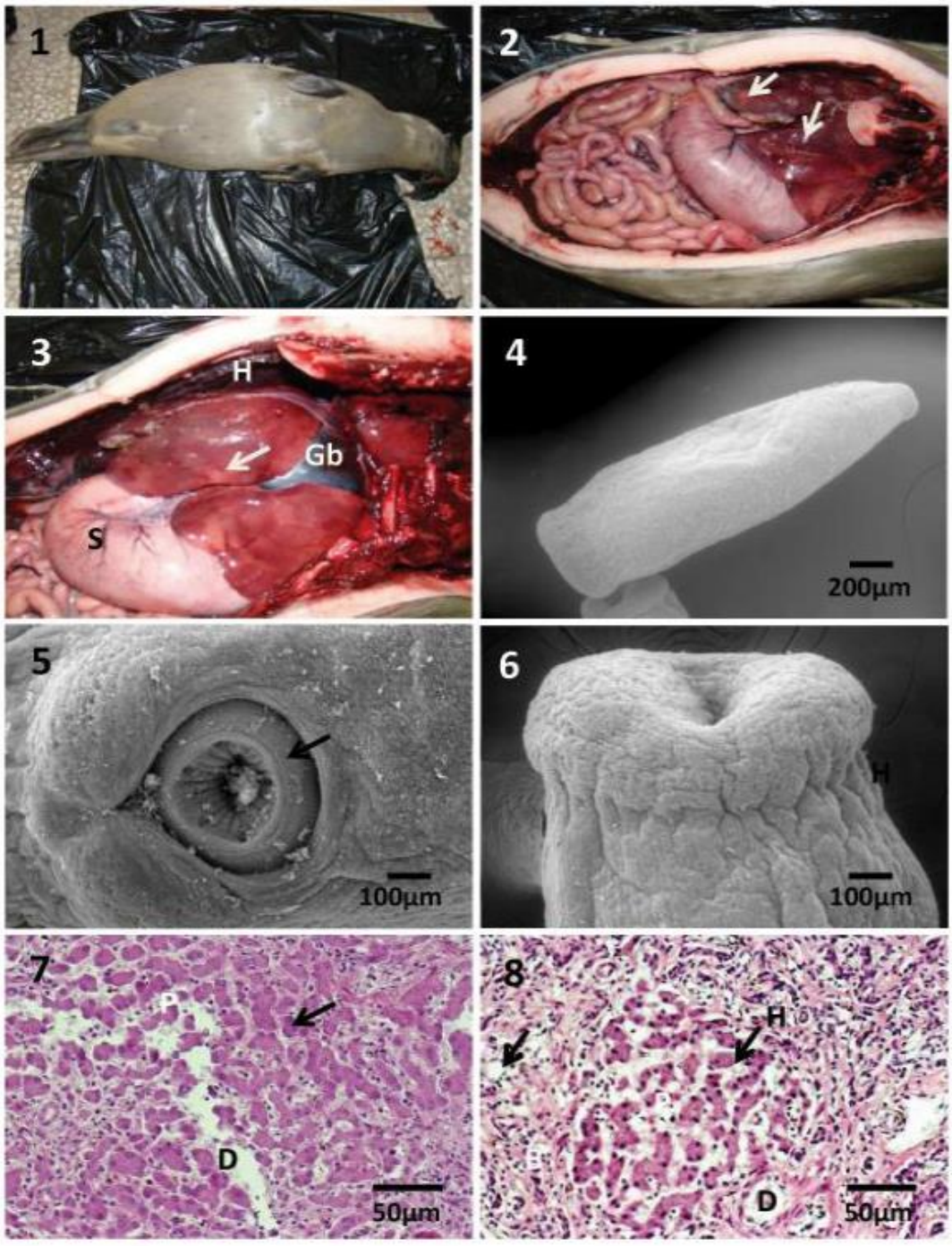

Fig. 1: Ventral view of the dead Caspian seal (*Pusa caspica*)/ **Fig. 2:** Internal organs of the Caspian seal, liver is pale in some areas/ **Fig. 3:** Internal organs of *P. caspica*. Note swollen gall bladder, "pooled" blood and Edematous foci on liver/ **Fig. 4:** Dorsal view of *Pseudamphistomum truncatum*. SEM micrograph/ **Fig. 5:** Ventral muscular sucker of *P. truncatum* / **Fig. 6:** Blunt posterior end of the trematode. Tegument spines are visible/ **Fig. 7:** Trematode path (P) invading hepatic lobule. Ducts and hepatocytes are visible/ **Fig. 8:** Normal hepatic lobule. Hepatocytes, bile ducts, and capillaries are visible

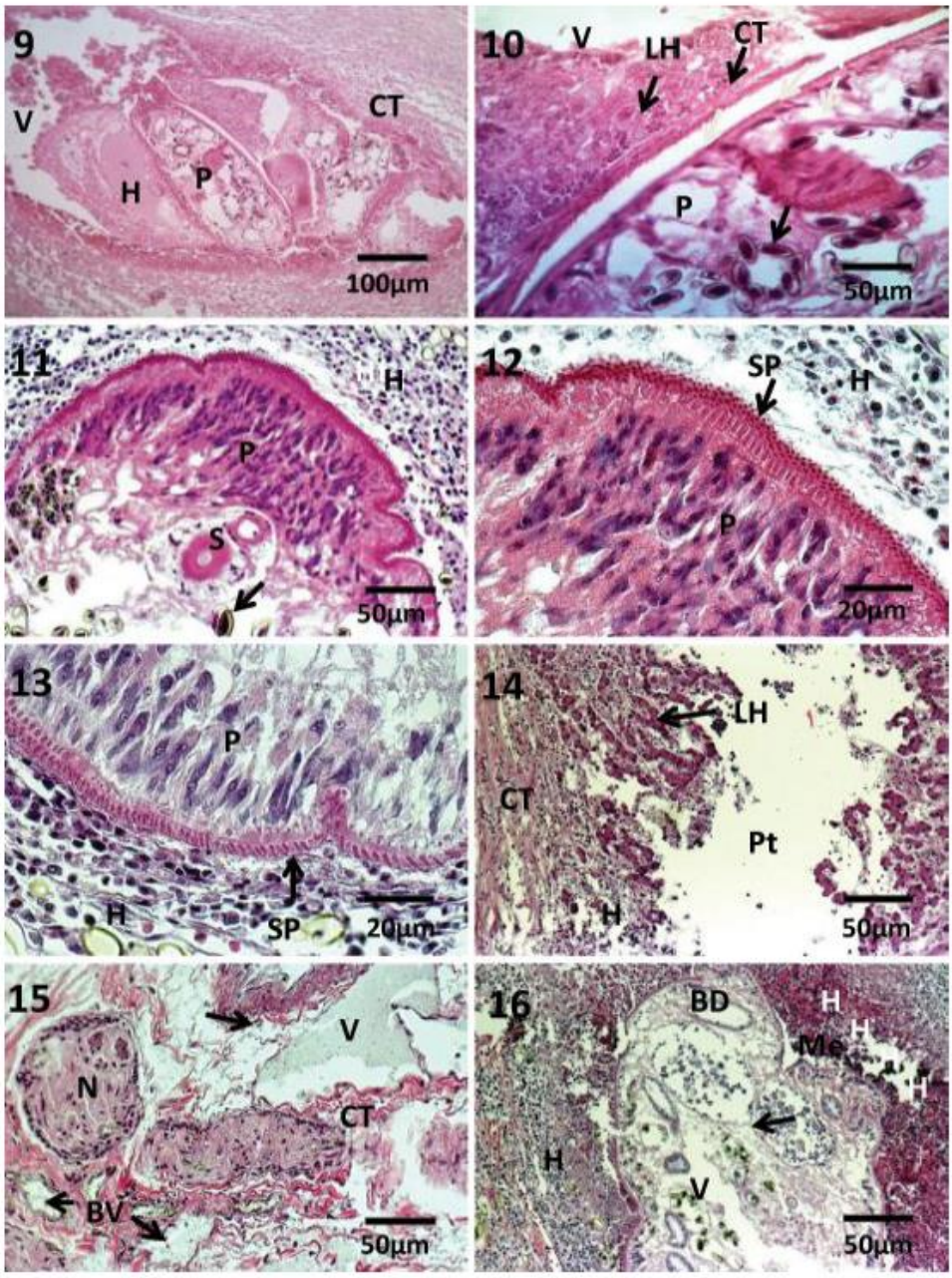

Fig. 9: Invaded hepatic lobule of the Caspian seal with the parasite (P) inside the liver tissue surrounded by vaculated areas, connective tissue and "pools" of blood / **Fig. 10:** Edge of the parasite (P) filled with eggs (arrow) and partially encapsulated (CT). Vaculated areas and necrotic liver hepatocytes (LH) next to the trematode/ **Fig. 11:** Edge of parasite (P) showing the tegument spines and sucker (S). Hemorrhage (H) next to parasite. Arrow shows trematode egg/ **Fig. 12:** Spines (SP) of the trematode (P). (H) is blood pooling/ **Fig. 13:** Prominent spines (SP) of the parasite (P)/ **Fig. 14:** Represents an area in the lives invaded by the parasite. Prominent pathway (Pt) of parasite with dislodged necrotic hepatocytes (LH), hemorrhagic areas (H) and connective tissue (CT)/ **Fig. 15:** Necrotic tissue and damage to the liver tissue due to the invading parasite, (V) vaculated areas, (CT) connective tissue, blood vessels and nerve tissue are visible within the hepatic lobule/ **Fig. 16:** Damage to the hepatic lobule due to the invading trematode, bile duct, (H) hemorrhaging, vaculated areas and the parasite pathway

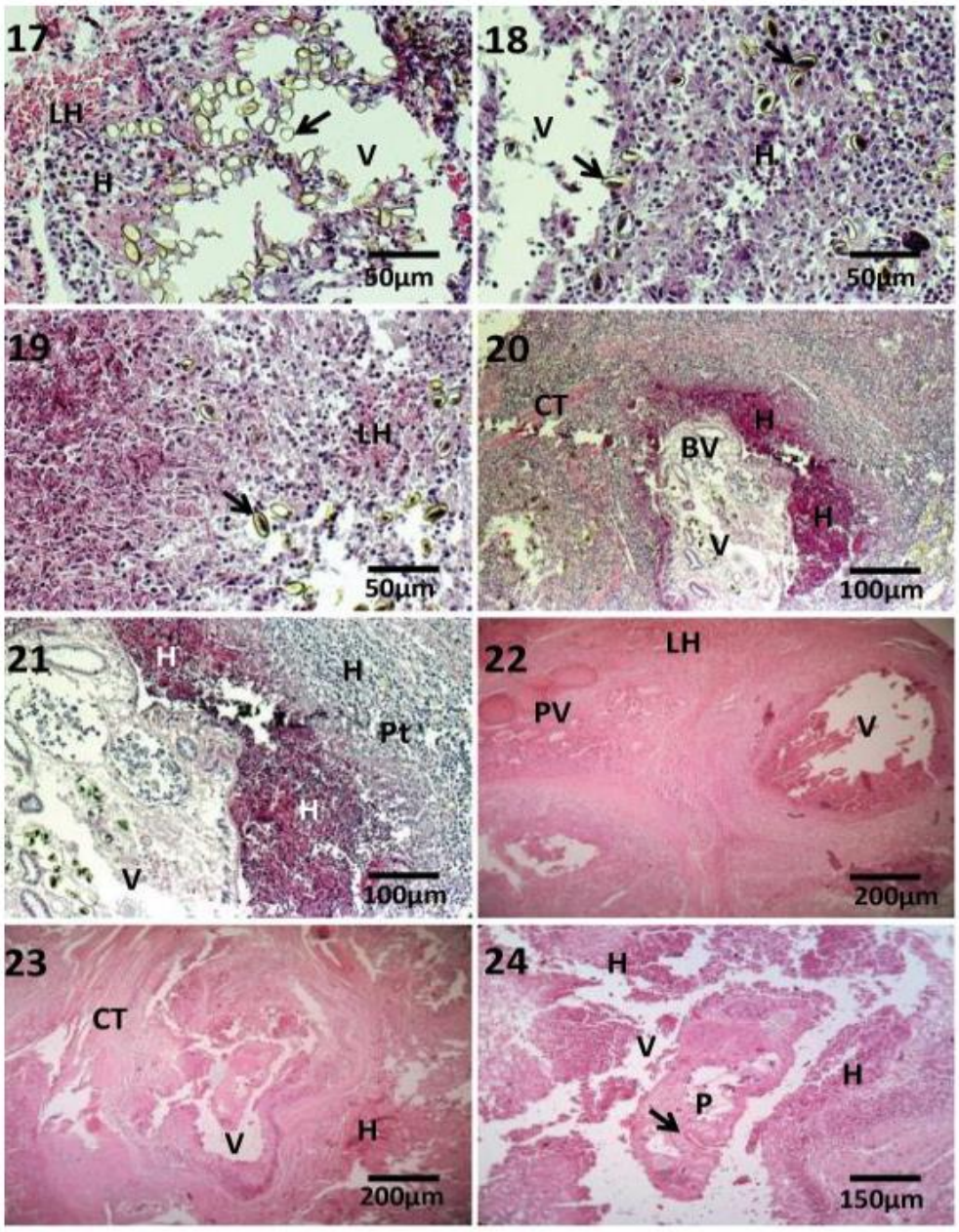

Fig. 17: Trematode eggs (arrow) scattered throughout the host liver lobule. Note vaculated areas, necrotic liver hepatocytes (LH) and hemorrhaging (H)/ **Fig. 18:** Eggs (arrow) with intact embryo's of the fluke. (V) vaculated areas and hemorrhage/ **Fig. 19:** Necrotic liver cells and hepatocytes (LH) as the invaded tissues. Eggs (arrow) of *P. truncatum* with intact embryo's/ **Fig. 20:** Extensive damage to the hepatic lobule. Note extensive blood loss (H), damaged blood vessels, and bile ducts in the vaculated areas. (CT) connective tissue/ **Fig. 21:** The invasive damage of the trematode is well expressed with extensive hemorrhaging (H) near the pathway of the parasite. Vaculated areas appear with damaged blood vessels and bile ducts/ **Fig. 22:** Invaded hepatic lobule (LH) with pooled blood (PV) and vaculated (V) regions/ **Fig. 23:** Remains of a damaged hepatic lobule with an influx of connective tissue (CT), vaculated regions (V), and hemorrhaging (H)/ **Fig. 24:** The parasite (P) visible within the damaged hepatic lobule. Necrotic hepatocytes and hemorrhaging around parasite

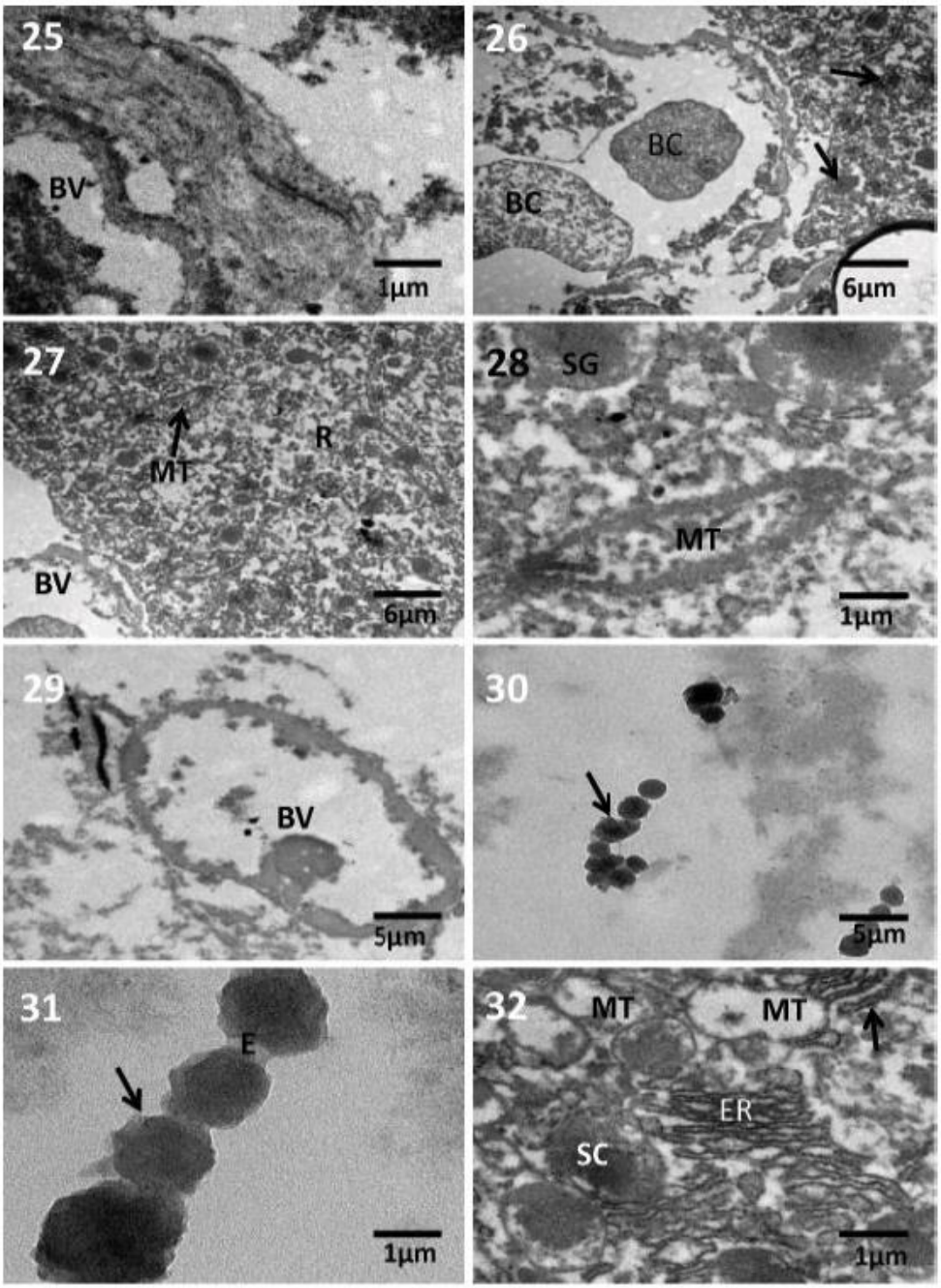

Fig. 25: TEM micrograph displaying the damaged blood vessel (BV) with surrounding organelles and collagenous fibers/ **Fig. 26:** Micrograph showing blood vessel with blood cells. Mitochondrion of a hepatocyte is also visible/ **Fig. 27:** Micrograph of a hepatocyte near a hepatic capillary (BV). Note the mitochondria (MT) and ribosomes (R)/ **Fig. 28:** High magnification of a hepatocyte mitochondrion (MT) with cristae. Secretory granules (SG) and endoplasmic reticulum near an organelle/ **Fig. 29:** Hepatic capillary (BV) with surrounding endothelial cells/ **Fig. 30:** Sphere shaped organisms (bacteria or virus) found within infected tissue of *P. caspica*/ **Fig. 31:** Higher magnification of sphere shaped particles/ **Fig. 32:** Organelles of a liver hepatocyte from the infected Caspian seal. (SC) secretory granule, (MT) mitochondria, (ER) endoplasmic reticulum (arrow)

Damaged hepatocytes and obstructed hepatic vessels and ducts are visible. There are numerous trematode eggs next to the parasite and within the symbiont. *P. truncatum* has a highly specialized outer tegument with numerous spines and lower fibrous and muscle layers (Figs. 10, 11, 12, 13). Parts of the posterior sucker and internal septums are visible. Extensive cell necrosis, hepatocytes and granulocytes are visible next to the invading trematode (Fig. 12, 13). The path of the parasite within the host hepatic tissue is very prominent (Figs. 14, 15, 16) with resultant cell necrosis, hemorrhaging and scattered islands of necrotic hepatocytes. Mononuclear host cells and granulocytes represent the remains of the hepatic lobule of the infected liver of *Pusa caspica*. The endothelial lining of host ducts and vessels can be seen on the stained sections surrounded by connective tissue and islands of loose red bloods cells. The host is unable to isolate the parasite with collagenous fibers (encapsulation).

One feature of the sections is the consistent presence of trematode eggs near the symbiont and throughout the surrounding tissue (Figs. 17, 18, 19). Many of the eggs are empty but others have embryonic contents of a larval trematode. Near the eggs, there is a collection of necrotic hepatic tissue with granulocytes and mononuclear cells. Hemorrhaging of host tissue is consistent with parasite presence and migratory activity (Fig. 20, 21). Figure 21 shows extensive blood loss, vaculation and potential connective tissue replacement. The parasites are actively migrating and the host is unable to encapsulate the invader. Bile ducts and blood vessels with the endothelial linings are prominent for the damaged areas (Figs. 21, 22). Figure 22 displays a vaculated area where the parasite once resided in the hepatic tissue. Figure 23 and 24 show the parasite and the necrotic damage of nearby tissue.

These latter figures displaying parasite damage should be compared to normal Caspian seal hepatic tissue (Figs. 7, 8). Common around the parasite is marked infiltration and migration of inflammatory cells, macrophages, heterophils, plasma cells and then the subsequent connective tissue cells and fibers in an attempt to encapsulate the invaders (Figs. 9, 11, 12, 13). This represents a typical multifocal granulomatous inflammation. There is always the presence of normal hepatocytes and ducts near the parasite. The hepatic sinusoids and ducts are often dilated and congested with necrotic debris.

TEM: The results of the TEM pathology of *P. truncatum* are displayed with figures 25 to 32. In general, these micrographs show the necrosis of cells as emphasized by organelle destruction (Fig. 32). Figure 30 and 31 show the invasion of an additional pathogen either a virus or cocci bacterium. There is a chain-like series of spheres interconnected by a fibrous material. These spheres were common in the viewed grids and further studies should be initiated to determine their identity. Figure 25 and 26 show membranous layers surrounding vessels (endothelium) with blood cells (erythrocytes) within the lumen (Fig. 26). Figure 27 and 28 display the organelle destruction of impacted hepatocytes as well as hepatic capillaries extending through the tissue. Figure 29 is a scan through a bile canaliculus which terminates in the bile duct. Endoplasmic reticulum, secretory granules and mitochondria are visible for Figure 32. The latter figure shows the destruction of the cristae of the mitochondria. Note the intact mitochondria for figure 26. The invasive trematode parasite (*P. truncatum*) is causing extensive cellular damage as displayed by the organelles.

Conclusion

It was shown that this trematode is quite invasive and pathogenic to Caspian Seal and as this host is the only mammal of Caspian Sea that is an endangered species, this needs more investigation toward control and possible treatment of this helminth that could help the conservation of this mammal.

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