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Research Article

Examination of Paraffin Sections of Different Rainbow Trout (*Oncorhynchus mykiss*) Tissues by Light and Scanning Electron Microscope

Beste Demirci[1], Funda Terzi[2] and Osman Sabri Kesbiç[3]*

[1]Department of Anatomy, Faculty of Veterinary Medicine, Kastamonu University, 37200, Kastamonu, Turkey

[2]Department of Pathology, Faculty of Veterinary Medicine, Kastamonu University, 37200, Kastamonu, Turkey

[3]Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Kastamonu University, 37200, Kastamonu, Turkey

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***Corresponding authors:** Osman Sabri Kesbiç, Associate Professor, Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Kastamonu University, 37200, Kastamonu, Turkey, Tel: + 90 366 280 5113; E-mail: osmankesbic@yahoo.com, okesbic@kastamonu.edu.tr

ORCID: https://orcid.org/0000-0002-1576-1836

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Abstract

The current study aimed to highlight histopathological findings in paraffin block sections of the liver, gill kidney, and pyloric cecum of rainbow trout (*Oncorhynchus mykiss*) by different imagining devices such as Scanning Electron (SEM) and Light Microscope (LM). To determine the performance of different imagining methods two different thickness paraffin sections such as 5 and 15 µm about various rainbow trout tissue were prepared for imagining different devices. That sections were imagined by SEM and LM, both sections including 5 and 15 µm were imagined by SEM while just 5 µm was an image by LM. In LM imagining, it was detected that hydropic degeneration and vacuole formations in the liver hepatocytes of fish, as well as hyperplasia in bile ducts. Lamellar epithelial cell hyperplasia/hypertrophy was mild and histopathological findings such as secondary lamellar elevation and edema were more severe in rainbow trout gills. Glomerular atrophy/hypertrophy was moderately detected in the kidneys and hydropic degeneration of tubular epithelium was more severe. No degeneration or necrosis was observed in the lamina epithelium of the pyloric cecum. In SEM imagining of different thickness paraffin sections, cartilage and secondary lamellar structure in the gills, glomerulus, and Bowman's capsule structure in the kidneys, and the structure of the pyloric cecum was observed. In the SEM imaging of the paraffin block sections of hepatocytes of the liver, the cell nuclei were determined, and also the grooves in the cytoplasm were thought to be vacuoles. As a consequence, the structural elements of the organ had higher clarity in SEM imaging from paraffin block sections, but the histopathological alterations remained unclear. As a result, SEM imaging of fish tissue is more suited for seeing tissue architecture, although LM imaging is better suited for determining and scoring histopathological variations.

Introduction

Aquaculture has become a highly productive industry for human consumption and animal protein production [1]. Rainbow trout (*Oncorhynchus mykiss*), one of the salmonids, is more preferred in this area due to its many features such as adaptation to the environment, reproductive efficiency, and disease resistance [2,3]. In scientific studies, organ anatomy, morphology, and histology of salmonid species are evaluated. Of these organs, the gills are anatomically located bilaterally on both sides of the pharynx in all fish. The gill filament is the basic functional unit or subdivision of the gill. Gill filaments, also called primary lamellae, consist of a series of pouch-like or arch-like structures that provide physical support [4]. Fish gills are an organ with many functions such as respiration, ion regulation, acid-base regulation, and nitrogenous waste excretion, and constitute more than 50% of the total surface area of the animal [5]. Salmonid and other fish species have morphologically and histologically all structural elements (liver cells-hepatocytes, blood vessels, and biliary tract) in their liver

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[6]. Fish liver morphology and histology, unlike mammalian liver structure, is the absence of its basic morphological unit, liver lobules, and portal triads [7]. Instead, fish hepatocytes display a diffuse or radial organization in the branching tubules that make up the liver parenchyma [8]. Additionally, the liver plays an important role in essential body functions such as regulation of metabolism, synthesis of plasma proteins, storage of energy, certain vitamins, and trace metals, conversion, and excretion of steroids, and xenobiotics [9].

The intestine is the main organ for digestion/absorption in fish and is critical for water and electrolyte balance, endocrine regulation of digestion and metabolism, and immunity [10]. Intestine histology consists of tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa of the intestinal wall. There are numerous villi and a simple columnar epithelium associated with the intestinal mucosa, goblet cells, and intraepithelial lymphocytes [11]. The pyloric caeca are quantitatively the most important part of the gastrointestinal tract concerning food digestion in salmonids [12,13]. The structure of the kidney in fish species is quite similar, and the kidneys of trout are fused, appearing as a single organ instead of two. Based on location as well as morphological differences, it is divided into a cranial or head kidney and a caudal or trunk kidney [14]. The histological structure of the salmonid kidney consists of a functional unit called the nephron, which includes the glomerulus, Bowman's capsule, proximal, distal, and collecting tubules [15]. The kidney is the primary organ for water elimination and ion reabsorption mechanisms in the kidney minimize ion loss in these fish [16].

In fish, biochemical, growth, and histopathology, along with other methods, are biomarkers used to evaluate the effects of both internal (feed used) and external (aquatic) environmental conditions [12,17]. Rainbow trout (*Oncorhynchus mykiss)* histopathology can be used to provide a prognostic diagnosis of potential pathophysiological effects and to create specific models of both acute and chronic detrimental effects on tissues and organs (gills liver, kidney, intestine, pyloric caeca). These histopathological data are scored semi-quantitatively and quantitatively for statistical analysis. Semi-quantitative scoring is still the most preferred by pathologists today for incorporating histopathological information into biomedical research.

Among the more established morphological research techniques such as light microscopy and transmission electron microscopy, scanning electron microscopy (SEM) is also preferred today, which allows the examination of large surface areas at high resolution and magnification [18]. SEM is widely used to study the structural details on the surface of biological samples [19]. In addition, SEM enables the distinction between inorganic nanoparticles and the surrounding organic structure and demonstrates nanoparticles in cells and tissue [20]. In fish, the morphology of the tongue [21], kidney [22], skeletal muscles [23] and spleen [24], was demonstrated using the SEM.

From that perspective, the study aimed to determine to performance of different imagining devices such as SEM and LM in rainbow trout tissue paraffin sections.

Materials and methods

In this study, fifty healthy rainbow trout (51.22 ± 3.04 g), which were used in a study approved by the Kastamonu University Animal Experiments Local Ethics Committee (Decision no: 2021-4/32), without any experimental application, were used. The fish were kept in the closed circuit experiment system with 10-15% water change daily, dissolved oxygen 8.4 ± 0.2 mg/L, temperature 13.8 ± 1.2 °C, and pH 7.7 ± 0.1. No lesion was found in the macroscopic examination of rainbow trout. A systemic necropsy of rainbow trout was performed. After necropsy, liver, gill, kidney, and pyloric cecum tissue were fixed in 10% formaldehyde solution.

Histopathological analysis

Liver, gill, kidney, and pyloric cecum tissues were trimmed and taken into the cassettes and a routine histopathological method was applied with the following procedure; (1) graded alcohol series from 70 to 96%, (2) xylol for 2h in 30 min. periods. Then, the tissues were blocked with paraffin, and sections of 5 µm and 15 µm thickness were cut on the rotary microtome (Leica RM 2255). The sections were stained with hematoxylin-eosin. Sections were examined under a light microscope (Leica DM 400B) and photographs of the sections were imaged by a camera attached to the microscope. Scores were scored semi-quantitatively as indicated by the severity and extent of changes; none (-) mild (+) moderate (++) and severe (+++) [12].

Scanning electron microscopy (SEM) analysis

Five and fifteen µm thick sections from paraffin blocks of the liver, gill, kidney, and pyloric cecum of rainbow trout were taken on polylysine slides. The sections were first kept in xylol series (10 minutes each) and then in alcohol series (100%, 96%, 80%, and 70%) for five minutes and dried at ambient temperature. First, sections were coated with gold-palladium (Au-Pd) (Cressington, Sputter Coater 108 Auto). The ETD detector was then examined and imaged under a high vacuum at 5.00 kV with a scanning electron microscope (FEI, Quanta FEG 250). SEM analysis was performed at Kastamonu University Central Research Laboratory, Imaging Laboratory.

Results

Histopathological results

Liver: Hydropic degeneration and vacuolization in the hepatocyte cytoplasm, and congestion and dilation with hyperplasia in the bile ducts were scored. Vacuole formation was found in 34 fish (+1; 5 fish, +2; 10 fish and +3; 19 fish) in the hepatocyte cytoplasm (Figures 1A-C). Hydropic degeneration in hepatocytes was detected as mild in 21 fish and moderate and severe in 10 fish. In addition, congestion and dilatation were severe in all fish. Kupfer cells are commonly seen with hyperplasia in bile ducts (Figure 1D), Table 1.

Gill: Epithelial cell hyperplasia/hypertrophy in the lamellar, fusion in the secondary lamella, degeneration in the lamellar epithelial cells, lamellar epithelial lifting and edema, and

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telangiectasis/aneurysm in the lamella were scored. Lamellar epithelial cell hyperplasia/hypertrophy was mild in 18 fish, moderate in 4 fish, and severe in 1 fish. (Figures 2 A-C). In addition, secondary lamella lifting and edema (Figure 2D) were found in more than half of the fish (82%).

Kidney: Atrophy in the glomerulus was found to be mild in 20 fish, and moderate and severe in 3 fish each. Glomerulus hypertrophy was determined in 37 fish (+1; 22 fish, +2; 8 fish and +3;7 fish). Hydropic degeneration of the tubular epithelium was observed in 44 fish with mild to moderate severity (Figure 3A-B) and severe in 3 fish. In addition, pycnosis was found in tubular epithelial cell nuclei.

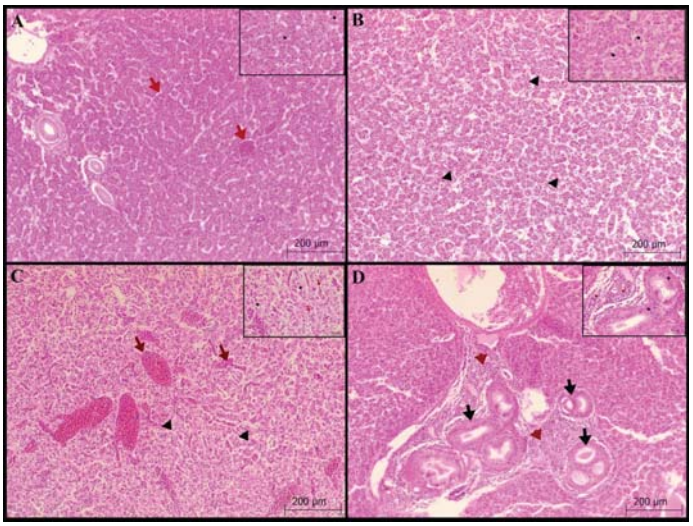

Figure 1: Hematoxylin-Eosin staining. Liver. A. Mild vacuolization (black arrows) and congestion (red arrows) in hepatocytes. Bar: 200 µ. B. Moderate vacuolization (arrowhead) in hepatocytes, congestion, and increase in Kupfer cells. Bar: 200 µ.C. Severe vacuolization, lipidosis (black arrows), and congestion (red arrows) in hepatocytes. D. Severe hyperplasia of the bile ducts and severe cell infiltration in the portal area. Bar: 200 µ.

Table 1: Semi-quantitative scoring of intestinal, liver, gill, kidney, and spleen histopathological lesions in rainbow trout.

Organs	Lesions	-	+	++	+++
Gill	Lamellar/filament epithelial cell hypertrophy/ hyperplasia	27	18	4	1
	Lamellar epithelial lifting/edema	9	13	8	20
	Hepatocytes hydropic swelling	9	21	10	10
Liver	Changes in cytoplasmic vacuolation	16	5	10	19
	Congestion dilated sinusoids	0	10	21	19
	Hyperplasia in the bile ducts	13	16	7	14
	Increase in Kupfer cell	2	20	18	10
	Hydropic swelling of cells in tubules	2	23	21	3
Kidney	Pycnose in tubulous cells	16	17	16	1
	Atrophy in Glomerulus	24	20	3	3
	Glomerulus Hypertrophy	13	22	8	7
	Infiltration in the intertubular area	0	0	3	47
	Necrosis of intestinal epithelial cells	50	0	0	0
Intestine	Congestion	22	24	4	0
	Cell infiltration in the Lamina Propria	44	6	0	0
	Cell infiltration in the submucosa	42	7	0	1

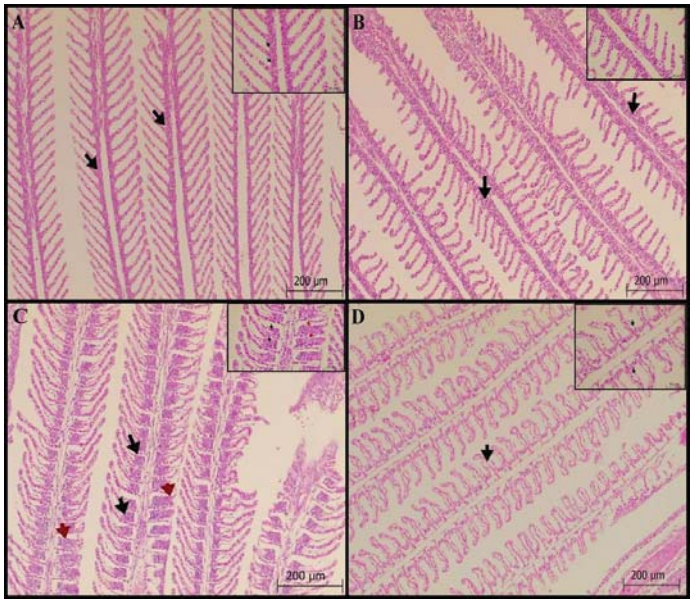

Figure 2: Hematoxylin-Eosin staining. Gill. A. Mild cell infiltration (Black arrows) in lamellae. Bar: 200 µ B. Moderate cell infiltration (Black arrows) in lamellae. Bar: 200 µ. C. Severe cell infiltration in lamellae and fusion (red arrows). Bar: 200 µ. D. Edema (black arrow) in the secondary lamellae and lifting Bar: 200 µ.

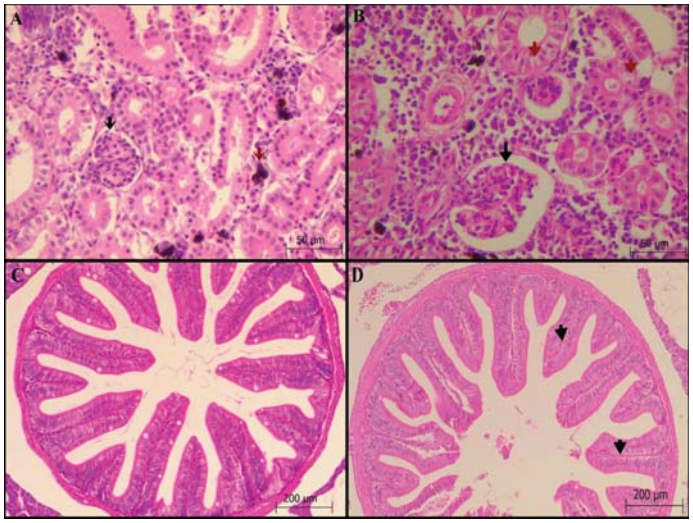

Figure 3: Hematoxylin-Eosin staining. Liver and pleric caecum A. Hypertrophy of the glomelus (black arrows) and melanin pigment (Red arrow). Kidney. Bar: 50 µ. B. Atrophy of the glomeruli (Black arrow) and hydropic degeneration of the tubules (red arrow). Kidney. Bar: 50 µ. C-D. Hyperemia in the lamina propria. Pleric cecum. Bar: 200 µ.

Pyloric cecum: No lamina epithelial degeneration or necrosis was observed. While no cell infiltration was detected in the lamina propria and submucosa, only mild hyperemia (Figure 3C-D) was observed.

SEM results

Paraffin block sections of 5 and 15 µm thickness taken from the gill, liver, kidney, and pyloric cecum of rainbow trout were demonstrated by SEM. In liver paraffin block sections, nuclei in hepatocytes and vacuoles in hepatocytes (Figure 4A-B) were determined. Gill cartilage and lamellar structure (Figure 5A) were seen. The kidney glomerulus structure and Bowman's

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capsule structure (Figure 5B) were observed in both 5 µm and 15 µm thick paraffin block sections. The histological structure of the pyloric cecum (Figure 5C) was photographed.

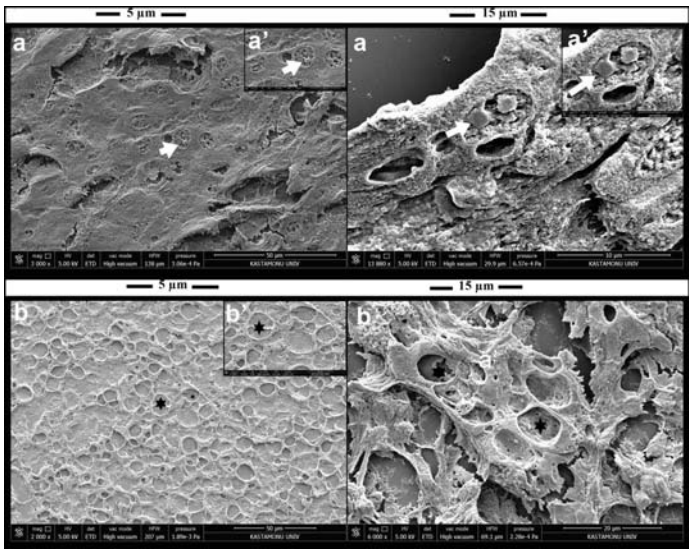

Figure 4: SEM image of paraffin block sections. Liver a. Cross-sectional area of the liver in 5 and 15 µm paraffin sections. Bar: 50 and 10 µm. a': Double nucleus appearance in hepatocytes (white arrows). Bar: 10 µm b. Cross-sectional area of the liver in 5 and 15µm paraffin sections. Bar: 50 ve 20 µm. b': Vacuole formation in hepatocytes (stars). Bar: 10 µm.

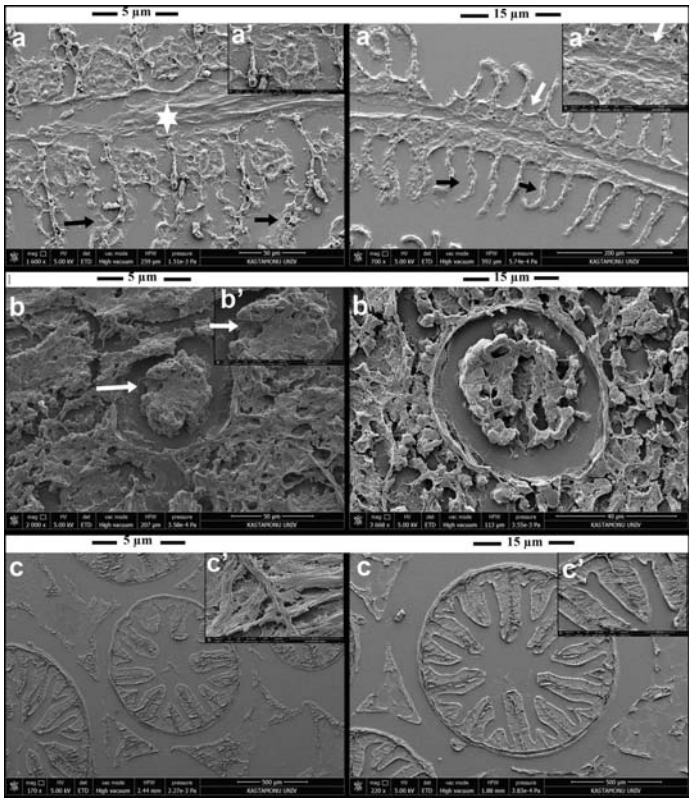

Figure 5: SEM image of paraffin block sections. a. Cross-sectional area of the gill in 5 and 15µm paraffin sections. Bar.50 ve 200 µm. a': Secondary lamellae (Black arrows) and cartilage (star). Bar: 20 µm. b. Cross-sectional area of the kidney in 5 and 15µm paraffin sections. Bar. 50 ve 400 µm. b': Bowman's capsule and glomerulus. (white arrows). Bar: 10 µm c. Pyloric cecum paraffin section face. Bar: 500µm. c': Appearance of villi. Bar: 30 µm and 100 µm.

Discussion

In the fish, many biomarkers (molecular, biochemical, physiological and histopathological, etc.) are used to determine the negative acute/or chronic effects of stress factors on tissues. Transmission Electron Microscopy (TEM) is used to determine the structure and changes of cell organelles, while Scanning Electron Microscopy (SEM), a surface imaging technique that provides a high depth of field, is used to view the 3D structure of cells [25,26]. This study, it was aimed to visualize the liver, gill, kidney, and pyloric caecum parts of rainbow trout in paraffin sections with light microscopy and SEM.

In SEM, tissue sample preparation is done by fixation, dehydration, drying, and conductive coating. Formaldehyde for Light Microscope, Glutaraldehyde (GA), and Osmium Tetroxide (OT) for SEM is used to fix tissue samples [27,28]. Nordestgaard and Rostgaard [29] determined that hepatocytes decreased in fixed volumes in the detection of glutaraldehyde and glutaraldehyde + OsO4. However, Maupin-Szamier and Pollard [30] state that osmium damages proteins and other components. The morphological structure of the gills in fish has been demonstrated in previous studies by Scanning electron microscopy [31,32]. Paruruckumani, et al. [33] determined the histopathological changes in the liver by SEM method with the detection of 2.5% glutaraldehyde and 1% osmium tetroxide in *Lates calcarifer* fish liver tissue. To demonstrate cell/tissue architectures by SEM in paraffin block sections, Sawaguchi, et al. (2018) examined rat organs from 30 µm thick paraffin blocks on New Silane II-coated microscope slides. In our study, we imaged paraffin sections of the liver, gill, kidney, and pyloric caecum with a Scanning electron microscope. We think that paraffin surface residues harm the detection of cells in SEM imaging. In addition, methods to completely remove paraffin should be developed for SEM imaging of paraffin block sections to be taken from archival materials.

The liver is the largest internal organ of the body and the organ where the nutrients absorbed in the digestive system are processed and stored for use by other organs of the body [34]. In the liver, hepatocyte vacuolization, fatty degeneration of the liver, changes in metabolic activity, changes in the liver parenchyma, and necrosis are the most common changes [6,35,36]. In our study, we detected hydropic degeneration and vacuole formations in the liver hepatocytes of fish, as well as hyperplasia in bile ducts. In our study, cytoplasmic vacuolizations in liver hepatocytes are one of the most prominent findings. We imaged fish liver paraffin sections by SEM and thought that the spaces in the cytoplasm of hepatocytes may be vacuoles in SEM imaging. The next generation of High-Resolution Field Emission Scanning Electron Microscopy (HRSEMs) not only has better performance characteristics but also has many applications that can be usefully used for diagnostic pathology and cell biology [37]. There is a need for studies using high-resolution SEM imaging techniques to detect pathological changes in liver hepatocytes.

In teleosts, the kidney, gills, and intestines are responsible for the excretion of body fluids and maintaining homeostasis [38]. In fish, the gills are an organ responsible for respiration

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and maintaining the optimal osmotic pressure and acid-base balance of body fluid [39]. In previous studies [40-44], rainbow trout gills were investigated histopathological lesions such as lamellar hyperplasia/hypertrophy, lamellar fusion, lamellar telangiectasia, and lamellar epithelial lift/edema, and lamellar clubbing. In fish, lifting of the lamellar epithelial cells from the basement membrane due to fluid penetration is the most common lesion and may result in decreased respiratory gas exchange (Salamat & Zarie, 2012). Hyperplasia and hypertrophy of epithelial cells and partial fusion of lamellae increase the distance between the blood and the environment and limit the entry of pollutants into the organism [45]. We found that while lamellar epithelial cell hyperplasia/hypertrophy was mild in fish, histopathological findings such as secondary lamellar elevation and edema were more severe. In the SEM imaging of the gills, the ultrastructural structure of the gills could be detected, but cell infiltration and edema that caused histopathological findings could not be detected.

The fish urinary system consists of the kidney, urethra, and bladder and plays a vital role not only in the excretion of urine but also in osmoregulation [46]. In fish, there is no metanephros formation, the pronephros and mesonephros are present as cephalic or head kidneys and exocrine or trunk kidneys, respectively [47]. In the head kidney, there is hematopoietic tissue, and this tissue is regarded as the teleost bone marrow [48,49]. In the trunk kidney, glomeruli, and proximal and distal tubules may be determined [50]. Researchers evaluate renal histopathological lesions such as enlargement of Bowman's space in kidneys, glomerular atrophy and hypertrophy, degenerative changes in tubular epithelium, necrosis and/or epithelial desquamation, protein in tubule lumen, and interstitial hematopoietic necrosis in fish. In our study, glomerular atrophy/hypertrophy was moderately detected in the kidneys of the fish. In addition, hydropic degeneration of tubular epithelium was more severe in fish. The histopathological evaluation of the kidney suggests that it will contribute to the studies to be carried out with creatures living in the aquatic environment. In addition, glomerulus and Bowman's capsule structure were determined in the imaging of kidney 5 and 15 µm paraffin sections with SEM, but the tubule structure could not be determined clearly. It is thought that studies should be done to show kidney structure in fish by SEM.

The general gastrointestinal morphology of fish relates to different dietary habits, including food content and frequency of food intake, as well as taxonomy, body size, and shape [52]. It promotes the absorption of nutrients, acting as a selective filter between the gastrointestinal tract (GI), lumen, and circulatory system, but also prevents the passage of harmful intraluminal xenobiotics [53]. In rainbow trout, the pyloric caecum is the site of digestion and absorption [54]. In the study, the pyloric cecum was examined and no lesion was found histopathologically, only hyperemia was detected in the lamina propria. In addition, the structure of the cells of the pyloric cecum could not be determined by SEM in paraffin incisions of the pyloric cecum, and it is thought that studies should be carried out to use special dyes to determine the cellular structures.

Conclusion

In rainbow trout, the structure of the liver, kidney, gill, and pyloric cecum on paraffin sections was demonstrated by SEM. In addition, it is thought that studies should be done to show the histopathological changes in fish species both in different thickness tissue and from paraffin block sections by SEM. As a result of the findings, SEM imaging is more effective in evaluating the structural differences of the tissues, while the conventional histopathologic method is more effective in evaluating and scoring the differences.

Declarations

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval and consent to participate: All the practices on rats were carried out with reference to European Union Directive and were approved by the local ethics committee of Kastamonu University (approval no:2021-4/32).

Authors' contributions: All authors participated in the design of the study, interpretation of the findings, and analysis of and review of the manuscript. BD: SEM imaging and concentration, FT: histopathological analysis and draft preparation; OSK statistical analyses and proofreading.

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