Chapter

Histopathology of Zebrafish (*Danio rerio*) in Nonclinical Toxicological Studies of New Drugs

Raphaelle Sousa Borges, Arlindo César Matias Pereira, Gisele Custodio de Souza and José Carlos Tavares Carvalho

Abstract

Zebrafish (*Danio rerio*) is a small-sized teleost fish natural of tropical regions, with a short life cycle and high homology toward humans. These features make zebrafish an attractive and promising model organism for nonclinical tests due to the ease of handling and cost–benefit compared to other models. The digestive, cardiovascular, urinary, nervous, and reproductive systems of zebrafish display feature similar to those of superior mammals, and due to its susceptible organs, the adult zebrafish has been used to test the toxicity of environmental compounds and potential drug candidates through histopathology analysis complementarily with other parameters. In such cases, the choice of the organ assessed relies on the type of compound tested, administration route, and biological activity. This chapter brings together histopathological nonclinical toxicity studies performed exclusively with zebrafish, highlighting significant histological changes found in its gills, liver, kidneys, and intestine. Based on the information presented here, it is expected that the researcher recognizes differences between healthy and changed tissue, without having to compare its result with other species.

Keywords: preclinical, histopathology, drug development, histology, zebrafish

1. Introduction

Zebrafish (*Danio rerio*) is a teleost fish from the family Cyprinidae, inhabiting fresh water of tropical regions. It is natural of Bangladesh, Nepal, and India and is popularly sold as an ornamental fish [1]. It has a small size and reaches up to 5 centimeters of length. The species often adapts to environmental changes of temperature and pH.

Since the first use of zebrafish as an experimental model in 1955, it has been consolidated as a model organism for biology, genetics, pharmacology, and general biomedical research. The exponential growth of zebrafish use in laboratories is due to several favorable features such as its fast life cycle (the adult stage reached about 6 months), high fecundity (hundreds of eggs are laid in each mating), transparency of the embryonic/larval stages which facilitates the observation of the development, low maintenance cost, and easy handling [2]. Due to its small size and low

body weight, it requires a relatively low quantity of the compound tested, which is an excellent advantage in the research [3]. Despite the phylogenetic distance, zebrafish has about 70% of genetic homology toward humans [4]; hence, it is considered a reliable tool for preclinical studies.

In pharmacology, determining the toxicity of the tested compound is a crucial step in preclinical studies since it will be the base for clinical trials when it is tested in humans. Zebrafish has been gaining attention for toxicological assays, either of administered compounds or those present in the environment due to its physiological response and histological features similar of those of mammals, potentially reducing the number of rodents used in the laboratory routine [2]. The sensibility of zebrafish organs to harmful compounds makes it possible to appraise through histopathology the safety of potentially bioactive substances [5]. Therefore, tissue changes are a useful tool to assess how a tested compound can exert a toxic in the animal; for this, it is essential to choose the most appropriate organs to be assessed [6].

The major organs accountable to metabolize and excrete xenobiotics are the liver and the kidney. The liver of zebrafish has disorganized hepatocytes and lacks Kupffer cells accountable to phagocyte foreign bodies [7]. However, despite these structural divergences, there are conserved aspects compared to the liver of mammals, for instance, the presence of hepatocytes and their main physiological processes performed through the action of the cytochrome P450, which makes it possible to compare hepatic lesions caused by nocive compounds in humans [8].

The kidneys have lymphoid, endocrine, and hematopoietic tissues. The latter is accountable to perform the functions of the bone marrow, absent in zebrafish. The renal filtration process contributes to the excretion of compounds previously metabolized in the liver. In zebrafish, both renal function and the glomeruli structure are similar to mammals, enabling the comparison of histopathology [9].

Zebrafish is an ideal model to assess environmental toxicity [10]. In these studies, the potentially toxic compounds present in the water are rapidly absorbed through the gills. Hence, the gills are an essential organ to perform histopathology when the fish absorb the compounds through immersion. Besides performing gas exchanges, the gills have a crucial role in the maintenance of osmotic balance, excretion of nitrogenated compounds, and acid–base balance of the adult fish.

The gastrointestinal tract of zebrafish, like those of mammals, has a mucous layer of simple columnar epithelial tissue formed by enterocytes arranged around the villi. Despite not having a stomach, the intestinal bulb—anterior portion of the intestine—substitutes this organ in its functions such as nutrient digestion and absorption. Therefore, histopathology of the intestine can be a useful tool to assess the safety of orally administered compounds [5]. Another difference compared to the gastrointestinal tract of mammals is the lack of Paneth cells and crypts of Lieberkuhn.

In the literature, the morphology of healthy zebrafish tissues is well known. However, to date, there are few studies dedicated exclusively to the comparison between healthy zebrafish and changed histopathology of the major organs of excretion and metabolization. Hence, this chapter aims to fill this gap in the literature, providing material for the researcher to better understand the histopathology of these organs in zebrafish, without needing to compare its results with other species. Also, the reader will learn the significant similarities and differences of zebrafish tissues compared to mammals, which is essential to interpret its results adequately.

2. Histopathology as a tool to assess toxicity in adult zebrafish

The potential of a chemical compound to induce injury to an organism is called toxicity. The toxic action depends on several factors such as the period of exposure,

mechanisms of transport, and target site interaction. One way to understand the effect of chemical agents in a living organism is the toxicity assays [11]. Toxicity assays are a critical step in the preclinical evaluation of new drugs; also, they are performed in environmental toxicity assessment of pollutants. For this, it is necessary to employ animal models able to extrapolate the results to humans posteriorly; hence, the animal chosen must have defined similarities with them, such as in the tissues and physiological response.

The study of tissue alterations is defined as histopathology. This method is useful to detect potentially deleterious effects of compounds to a particular organ or tissue [6]. The choice of the organ depends on the type of compound tested and the metabolism of the animal used. Zebrafish is an excellent model to perform histopathology assessment [12]. The genetic homology and conserved aspects compared to humans can be extended to disease phenotypes to perform comparisons [2]. Several organs of zebrafish have been used in toxicological studies, such as the gonads, pharynx, thyroid, intestine, liver, kidney, gills, and muscles. A considerable advantage of zebrafish in histopathology is the possibility to observe several organs in only one slide due to its small size.

Histopathology of zebrafish gonads was capable of showing the detrimental effects of chronic treatment of bezafibrate in gonad steroidogenesis and spermatogenesis. The bezafibrate—drug used in the control of cholesterol levels in humans—was administered orally in male zebrafish. The histological analysis showed testicular degeneration, and this could improve our understanding of the possible risks of prolonged bezafibrate use in the reduction of fertility in humans [13].

The gonads were also used to evaluate the long period of treatment with the hydroethanolic extract from *Acmella oleracea*, the "jambú," in adult zebrafish. This plant is popular in cooking from the north of Brazil and is used as an aphrodisiac agent. The results showed low toxicity, evidenced by few tissue changes in the ovaries and testes [14].

The muscle of zebrafish was used to perform histopathological analysis on the toxicity of *Streptomyces* sp. AKS2, a potential antibacterial against *Klebsiella* sp., injected intramuscularly. The tissue did not have changes, suggesting low toxicity. This study could point further uses of the muscle histopathology in future toxicity assays [15].

Despite often used to assess the effect of chemical compounds in the endocrine system, the histopathology of the thyroid has been reported less in fish models. Schmidt et al. [15] reported that sublethal concentrations of propylthiouracil and perchlorate induced cell changes in the thyrocytes, evidencing the efficacy of zebrafish thyroid tissue histopathology in toxicity assays. Moreover, histopathology analysis of thyroid tissue in male zebrafish was essential to assess the toxic effect of prolonged exposure to fluoride in this organ [16].

The gills are crucial to assess the toxicity of chemical compounds suspended in the water in environmental toxicity studies. Female zebrafish had few tissue alterations in the gills when exposed to silver nanoparticles [16]. However, in the presence of uranium, the gills, gonads, and muscles were affected, demonstrating the dangers of the contamination with this element [17].

In a study assessing the toxic effects of thallium—chemical element emitted mainly after combustion of mineral coal, easily incorporated to the soil and water—the gills were injured even at the lowest concentrations [18]. In another study, zebrafish exposed to cobalt chloride showed relevant tissue alterations in the gills, pharynx, and intestinal mucosa. The authors stated that adult zebrafish could provide a robust model to assess indicators of toxicity from chemical products [19]. Overall, these studies indicate that the histopathology of zebrafish gills can be a reliable model to assess whether a chemical compound is toxic.

As for the liver, there are relevant similarities between the liver of zebrafish and those of superior mammals in the most basal aspects and regulatory mechanisms related to hepatoxicity [19]. Due to its role in animals, the liver is essential to assess the metabolism of xenobiotic compounds.

Chronic administration of the fungicide boscalid induced histological injury in liver and kidney of zebrafish, hampering the metabolism of carbohydrates and lipids and the healthy development of the animals [20]. Moreover, in a study in which the fishes were exposed to tris(1,3-dichloro-2-propyl)phosphate (TDCPP), the liver of the animals had increased size and tissue alterations, showing the sensibility of this organ and its role in detoxification [21].

After subacute exposure to pharmaceutical products—such as carbamazepine, fenofibric acid, propranolol, sulfamethoxazole, and trimethoprim—the tissue changes in the liver varied according to the sex, being more prevalent in male fishes, whose the liver tended to increase and have more tissue alteration, reinforcing the importance of controlling the sexes in toxicological studies [22].

The females also can be used; in a study using female zebrafish exposed to sodium fluoride (NaF), the fishes had tissue degeneration [22]. In another study, long-term exposure to perfluorooctane sulfonate (PFOS) induced accumulation of lipid droplets in the liver of males and inhibited the growth of the gonads of female zebrafishes, suggesting a potential to induce malformation in embryos from exposed fishes [23].

The kidney and intestine have a role in the excretion of substances and are valuable to assess the toxicity of them. For instance, the intestine of adult zebrafish was used to assess the impact of silver nanoparticles coated with citrate [24] and cadmium present in the environment [25]. In the latter, the results showed a nonlinear response to the toxic effects, suggesting that the animals used may have developed a defense mechanism against the toxicity related to hormesis.

A method to quantify the tissue changes in zebrafish organs is through the histological alteration index (HAI). This method takes into consideration the average tissue changes based on a list of possible changes classified into three levels of severity. The method was first conceived for gills and, posteriorly, was adapted to the liver, kidney, and intestine, due to their sensibility to injuries caused by toxic agents [5, 9, 16, 26].

3. Main histological changes found in zebrafish organs after exposure to toxic compounds

In this section the major tissue changes caused by potentially toxic compounds observed in the gills, liver, kidney, and intestine and the possible physiological response that cause them will be presented. Pictures will be shown to compare healthy and changed tissues.

3.1 Gills

Zebrafish has four gill arches in each side of the pharynx; each of them has two rows of filaments, which in turn has secondary lamellae in both sides [11]. Firstly, the water enters through the oral cavity; then, it passes through the pharynx and gills, where the gas exchange occurs. The water is pushed through contraction and relaxation of the vestibular muscle and the operculum movements. The primary lamellae (gill filaments) are in the central portion of the gills formed by cartilaginous tissue supporting the venous sinusoids [7, 27].

Besides being accountable to perform the gas exchange in the water, the gills also have a role in the excretion of residues [27]. This organ is highly sensitive to toxic

compounds, and when exposed to them, it has typical tissue changes. One of the first alterations observed is the displacement of the lamella epithelial cells, which, according to Carvalho et al. [5], can indicate an adaptation attempt in the aquatic animal to the ongoing new environmental conditions. The space between the lamellae and the displaced epithelial tissue is filled with water, forming edema, which can hamper the function of this organ, suffocating the animal [28].

The change in the function of the gills can lead to an increase in size in the epithelial cells (hyperplasia). As a consequence of hyperplasia, the fusion of secondary lamellae can occur [29]. This feature blocks the passage of water and blood to decrease the work overload of lamellae cells. However, it causes a deficit of oxygenation and can induce the death of the animal [30].

Chloride cells are located on the basis of secondary lamellae and are accountable to pump sodium and chloride ions into the fish to maintain its osmotic balance [27].

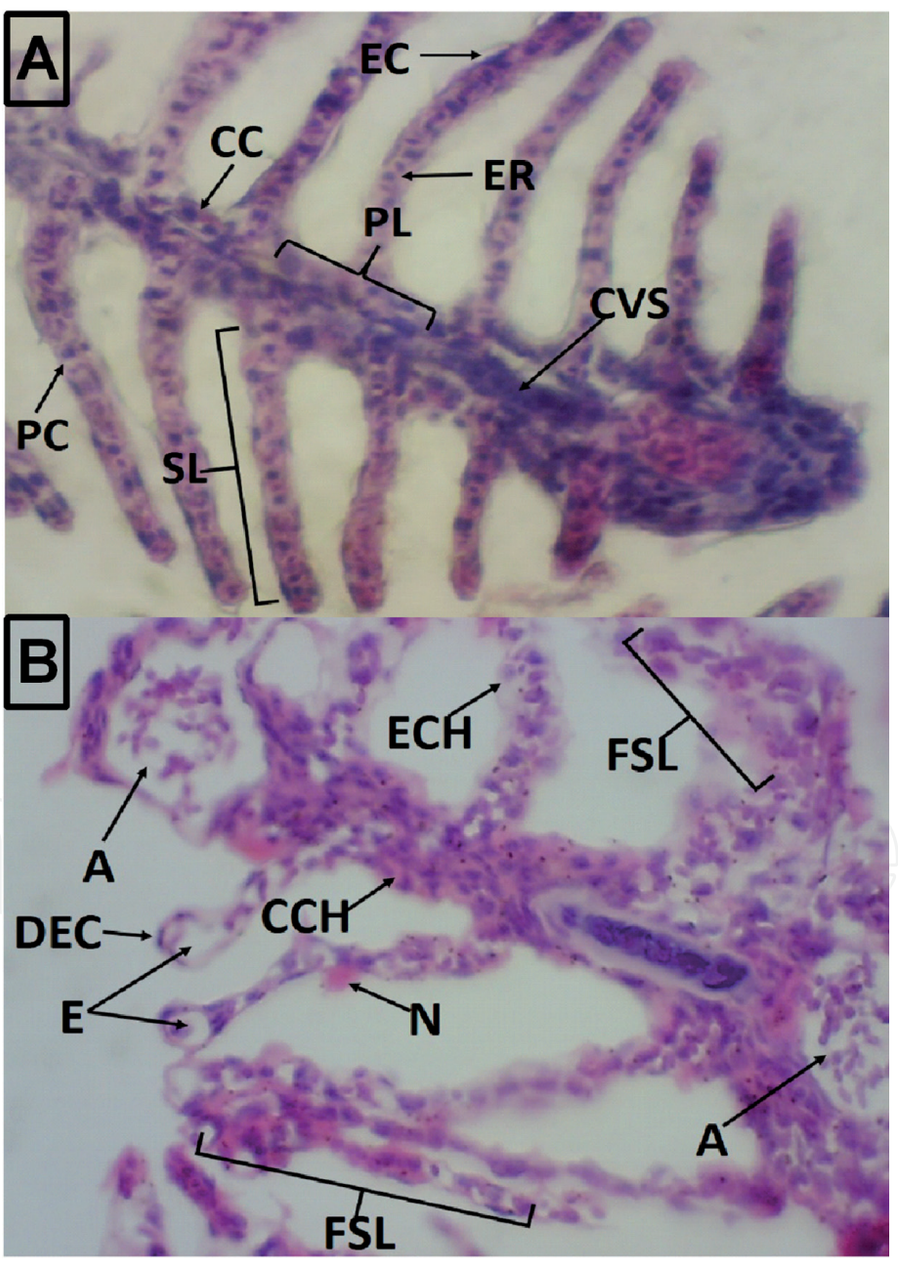

Figure 1.
Comparison between normal and changed gill tissues. (A) Healthy gill tissue, where PL is the primary lamellae; SL, secondary lamellae; CVS, cartilage supporting the venous sinusoids; EC, epithelial cells; ER, erythrocytes; PC, pillar cells; CC, chloride cells. (B) Changed gill tissue with typical histopathological changes, such as a, aneurysm; DEC, displacement of epithelial cells; ECH, epithelial cell hyperplasia; E, edema; FSL, fusion of lamellae; CCH, chloride cell hyperplasia; N, necrosis (H&E, ×40).

Hence, hyperplasia in these cells may indicate an osmotic imbalance in the fish, as the organism tries to adapt through the increase of sodium and chloride transport into the blood to reestablish the homeostasis [31].

Another essential cell in the maintenance of blood circulation is the pillar cell. Changes in its regular function can induce lamellae degeneration and blood flow dysregulation [32]. Progressive distension of the pillar cells can cause aneurysms, hemorrhages, and gill tissue collapse through all lamellae [5]. High toxicity-induced cell degeneration in the lamellae causes loss of tissue function, and hence, if necrosis is observed, the dose of the compound tested is highly toxic. Healthy gill tissue and significant tissue changes caused by toxic compounds cited in this section are shown in **Figure 1**.

3.2 Liver

The hepatocytes are the primary cells of the zebrafish liver and form the hepatic cords. In zebrafish—like in superior mammals—these cells act in the processing of proteins, carbohydrates, lipids, and vitamins and detoxification of xenobiotic compounds. The hepatocytes are accountable to synthesize the bile, which is transported through the bile ducts. Blood vessels are frequent and abundant in the liver [7, 27].

A common tissue alteration found in the hepatocytes is cytoplasm vacuolization due to a decrease of glycogen stores and lipid accumulation, which could be due to the action of toxic agents. This process is believed to hamper the normal function of the liver [9]. However, the decrease of glycogen stores should not be considered alone since due to the small size and high agility of zebrafish, zebrafish has an accelerated metabolism which can consume the glycogen if the animal is active enough [5, 29].

Changes in the nuclei morphology, such as vacuolization and atrophy, are often observed when functional alterations occur in the hepatocytes and can precede pyknosis (reduction and rounding of the nuclei that precedes apoptosis) and cellular degeneration. On the other hand, hypertrophy of the nuclei indicates intense metabolic activity in the hepatocytes, which can be caused by exposure to toxic agents [33]. When the hepatocytes degenerate, a relative reduction in the frequency of nuclei is observed, which is believed to be a defense mechanism.

The bile excretion also can be affected by toxic agents, causing cholestasis, which is an indication of liver dysfunction. Histopathologically, this is detected through the presence of brownish pigment inside the cells and is attributed to the failure in the excretion of bile pigments due to decreased capacity of bonding between bilirubin and glucuronic acid [29].

Other frequent changes observed are the increased frequency of blood vessels and hyperemia, mechanisms triggered to increase the blood flow to the liver, consequently increasing the supply of oxygen and nutrients in the affected area, preventing hypoxia [5, 9, 33]. Finally, if the animals are exposed to high levels of a toxic agent, rupture of blood vessels and tissue necrosis can occur. The tissue changes of the liver cited in the section and the normal histology of the organ are shown in **Figure 2**.

3.3 Kidneys

The kidney of zebrafish is accountable to excrete the excess of water that enters through the mouth; it also has a role in the filtration of residues and osmotic balance [9, 27]. This organ is one of the most affected by toxic compounds and has conserved similarities compared to those of mammals [5]. The nephrons are formed by glomeruli and distal and proximal tubules; the glomeruli are surrounded

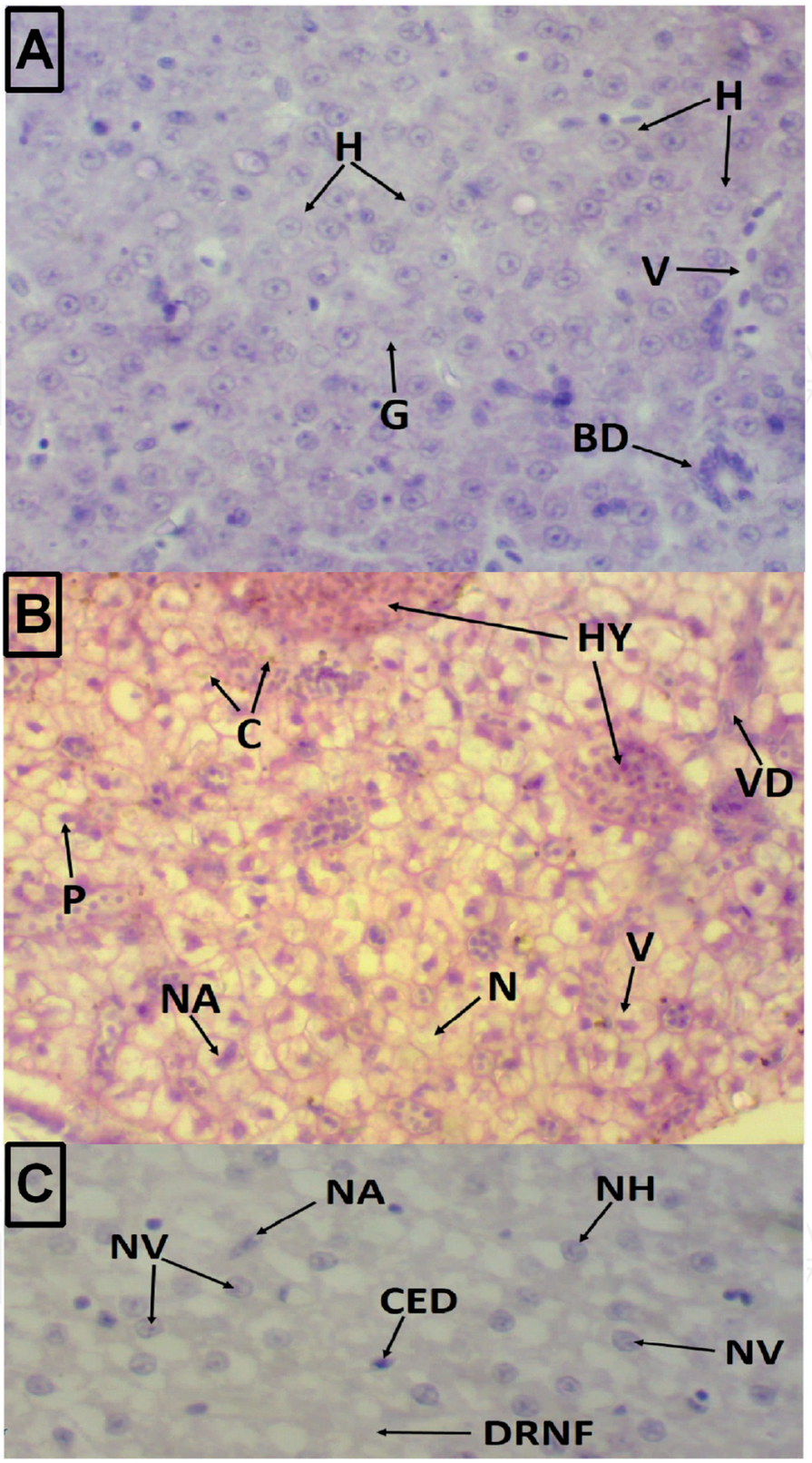

Figure 2.
Comparison between normal and changed liver tissues. (A) Healthy liver tissue, where H is the hepatocytes; V, blood vessels; BD, bile ducts; G, glycogen. (B) and (C) Changed liver tissue with typical histopathological changes, such as HY, hyperemia; V, vacuolization; P, pycnosis; C, cholestasis; N, necrosis; VD, vessel degeneration; NV, nucleus vacuolization; NA, nucleus atrophy; NH, nucleus hypertrophy; CED, cellular degeneration; DRNF, relative decrease of nuclei frequency (H&E, ×40).

by the Bowman capsule, while the tubules are surrounded by epithelial cells. Hematopoietic, interrenal, and chromaffin cells [7] are also observed in the interstice of the kidney.

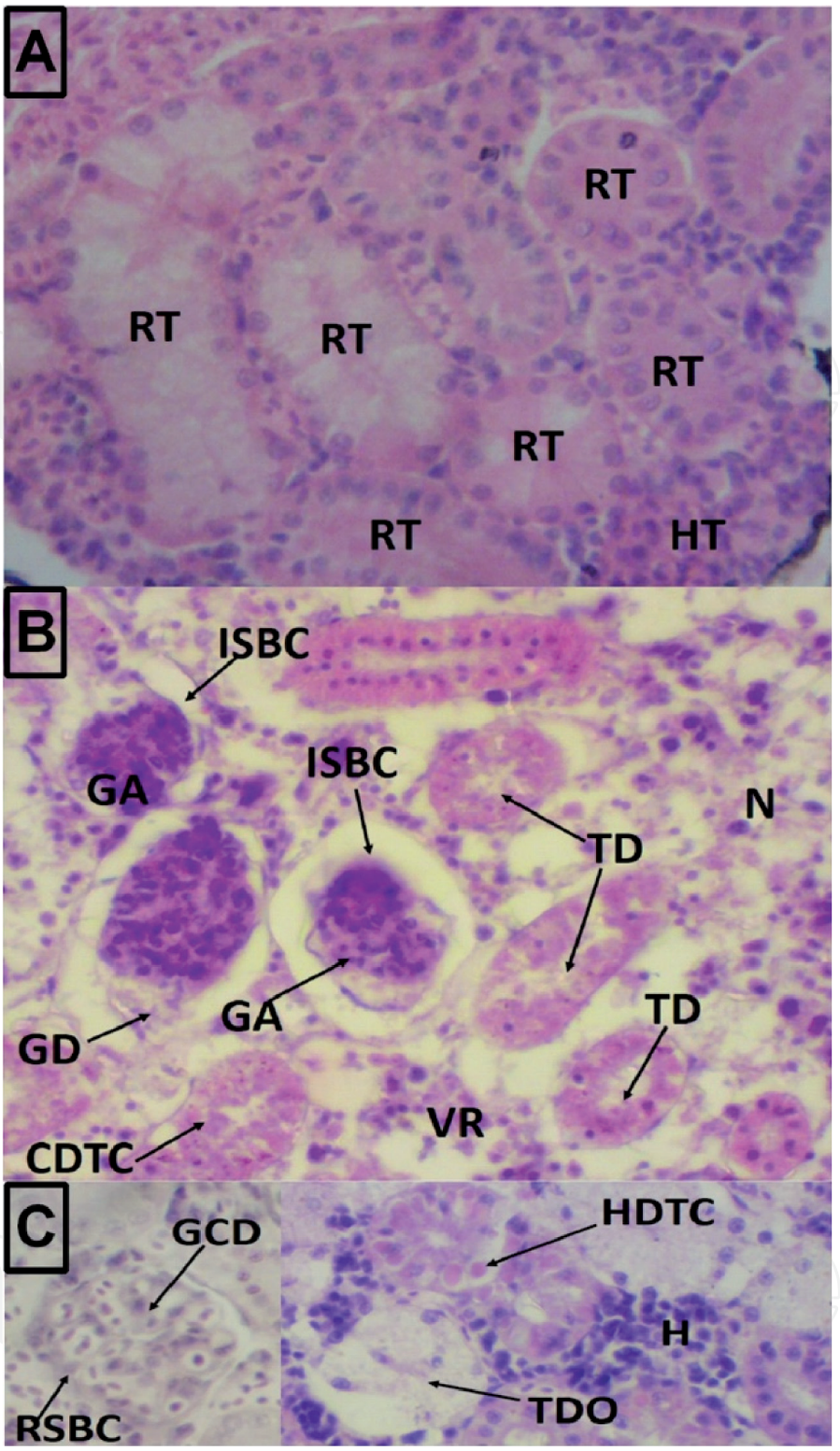

Figure 3.
Comparison between normal and changed kidney tissues. (A) Healthy kidney tissue, where RT is the renal tubules; HT, hematopoietic tissue. (B) Changed kidney tissue with typical histopathological changes, such as GA, glomerular atrophy; GD, glomerular degeneration; ISBC, increased space of the Bowman capsule; CDTC, cytoplasmatic degeneration of tubular cells; TD, tubular degeneration; VR, vessel rupture; N, necrosis. (C) Changed kidney tissue with GCD, glomerular capillaries dilation; RSBC, reduced space of the Bowman capsule; HDTC, hyaline degeneration of tubular cells; TDO, tubular disorganization; H, hyperemia (H&E, ×40).

The interrenal cells have an endocrine role functionally equivalent to the mammals' adrenal cortex and are accountable to synthesize cortisol in response

to physiological stress. On the other hand, the chromaffin cells are functionally equivalent to the adrenal medulla and synthesize epinephrine and norepinephrine, which will act in the cardiac rhythm [7].

In contact with toxic compounds, the most frequent tissue alterations of the kidney are found in the tubules. The hypertrophy of tubule cells is an alteration caused by dryness of the epithelial cells surrounding the tubules, which is caused by alterations in the filtration of the kidney. Frequent structural tissue alterations in the tubular cells include tubular disorganization, tubular degeneration, and cytoplasmatic degeneration. According to Carvalho et al. [5], these tubular alterations found in the kidney of zebrafish can be caused indirectly by metabolic dysfunction caused by toxic compounds.

When tubular cells are functionally changed, hyaline degeneration can occur, evidenced by the occurrence of eosinophilic granules their cytoplasm [5]. Hyaline degeneration can be caused by reabsorption of protein compounds excessively synthesized by the glomeruli [33].

Toxic compounds can also affect the function of the kidney glomeruli. In some cases, this can cause glomerulus atrophy or degeneration evidenced by the increase in the Bowman capsule space. In other situations, dilation of the glomerulus' capillaries can occur, which is a mechanism to reduce tissue damage by increasing the blood flow to the area.

The reduction of the Bowman capsule space can compromise the renal filtration, while the over increased hyperemia can potentially induce rupture of blood vessels [5, 29]. Both of these tissue changes can result in kidney necrosis [33]. The tissue changes often observed in the kidney of zebrafish are shown in **Figure 3**.

3.4 Intestine

The intestine of zebrafish is formed by villi, surrounded by a mucous layer. In the center of these villi is the lamina propria, where defense cells are found. Along the villi, there are the mucus-producing goblet cells and the enterocytes, accountable of nutrient absorption in the epithelium; the enterocytes also have a role in the immune response and osmotic balance [5, 9, 27]. Exposure to toxic compounds can damage the intestinal mucosa and hamper the cellular development in this tissue, causing a disturbance in its physiology evidenced by histological changes [5, 33].

A typical feature of injury caused by noxious compounds in the intestine is inflammation in the lamina propria. Inflammation is characterized by leukocyte infiltration, mainly neutrophils, with a morphology similar to mammals (clear cytoplasm and multi-lobed nuclei). Other inflammatory cells of zebrafish are the lymphocytes and monocytes, also similar to those of mammals [7].

The infiltration of eosinophils in the tissue caused by inflammation corroborates to the increased hyperemia [5, 34]. When stained with hematoxylin and eosin, these cells have a deeper pink-colored cytoplasm and a rounded peripheral nucleus [7]. A defense mechanism to avoid injury caused by leukocyte infiltration in the villi is the separation of lamina propria, which reduces the contact between the inflammatory cells and the enterocytes.

Another feature observed in the enterocytes after exposure to highly toxic compounds is vacuolization, which can be accompanied by edema. This latter is often associated with degeneration of the muscle layer and the villi. These tissue changes hamper the process of nutrient absorption and often precede necrosis [5, 33]. In **Figure 4** the typical intestine structure and the main tissue changes cited in this section are shown.

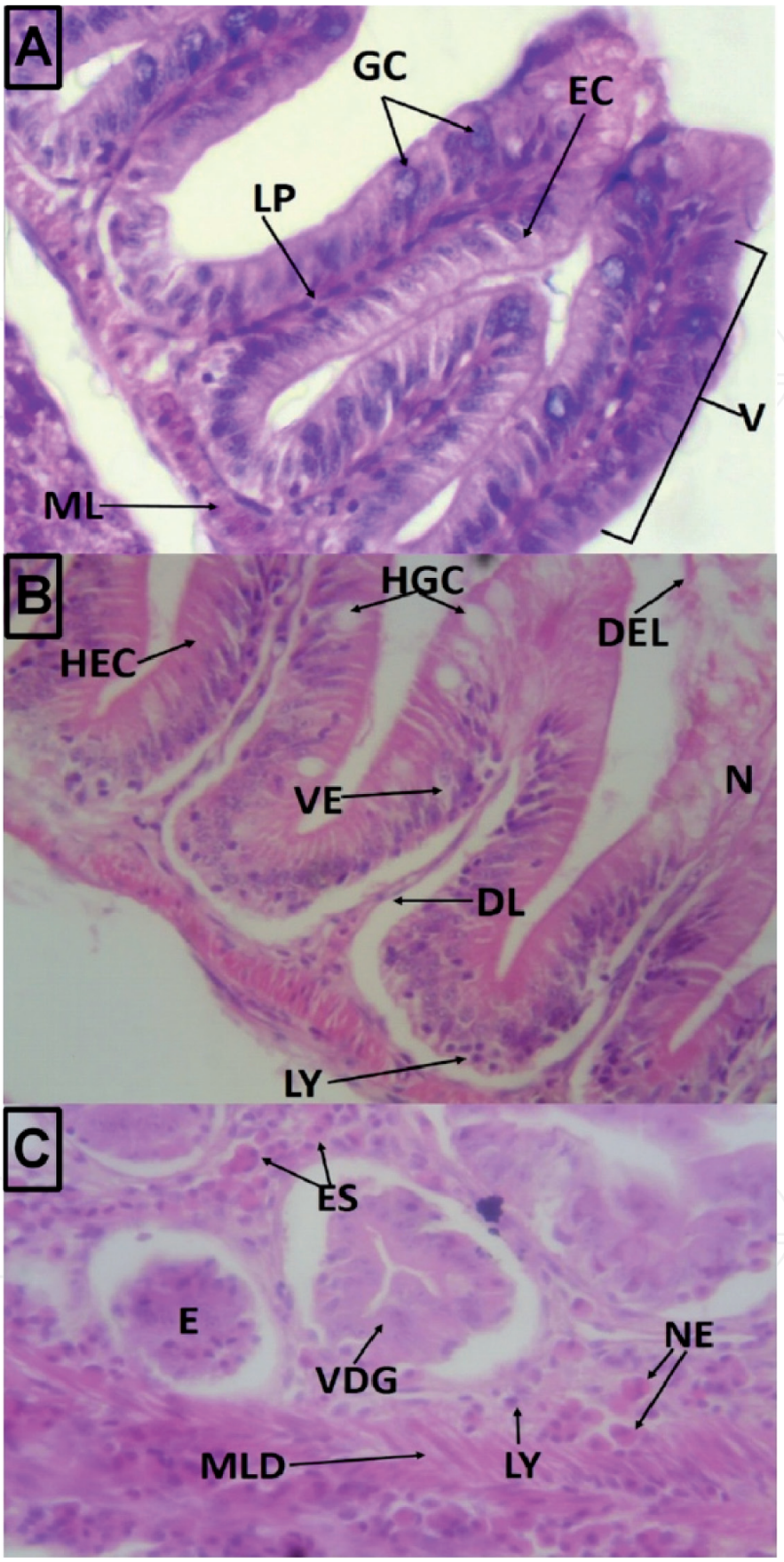

Figure 4.
Comparison between normal and changed intestine tissues. (A) Healthy intestine tissue, where V is the villi; LP, lamina propria; EC, enterocytes; GC, goblet cells; ML, muscle layer. (B) Changed intestine tissue with typical histopathological changes, such as HEC, hypertrophy of enterocytes; HGC, hyperplasia of goblet cells; VE, vacuolization of the enterocytes; DL, displacement of the lamina propria; DEL, detachment of the epithelium; LY, lymphocytes; N, necrosis. (C) Intestine lamina propria inflammation, with NE, neutrophils; LY, lymphocytes; ES, eosinophils; E, edema; VDG, villi degeneration; MLD, muscle layer degeneration (H&E, ×40).

4. Conclusion

This chapter, written by researchers of pharmaceutical and biological sciences, is intended for college students, postgraduation students, and researchers in the fields of biomedical science and related specialties. It is expected to aid researchers in the field of histopathology and toxicology to better understand the application of zebrafish in these areas. Relevant information about tissue changes are gathered caused by exposure to toxic agents in zebrafish and its potential application as a model in nonclinical toxicological studies. In this chapter previously unpublished pictures are presented that can serve as a primer for the study of healthy and changed tissues of gills, liver, kidney, and intestine of zebrafish.

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